UNIVERSITY OF EDINBURGH

THESIS

on

"THE CHEMISTRY OF ENSILAGE AND OTHER STUDIES"

submitted

by

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10. Fermentation studies on inoculated herbages. J. Sci. Food Agric. 1964, 15, 429.

11. The determination of water soluble carbohydrates in grass. J. Sci. Food Agric. 1964, 15, 395.

12. Fermentation studies on red clover. J. Sci. Food Agric. 1965, 16, 549.

13. A silica gel chromatographic procedure adapted to liquid-scintillation counting of ^{14}C labelled organic acids from plant material and silage. J. Sci. Food Agric. 1966, 17, 257.

14. The buffering constituents of herbage and of silage. J. Sci. Food Agric. 1966, 17, 264.

15. The effect of temperature on ensilage. J. Sci. Food Agric. 1966, 17, 476.

16. Changes in organic acid composition during incubation of aseptically-grown grass. J. Sci. Food Agric. 1967, 18, 19.

17. The principles of ensilage. Z. Tierphysiol. Tierernahr. Futtermittelk. 1966, 21, 103.

18. Some biochemical and microbiological aspects of ensilage. In the press, 1967.

19. Losses during ensilage. In the press, 1967

20. Chemical changes and losses during the ensilage of wilted grass. In the press, 1967.

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21. Application of magnesium sulphate to grass for silage as a means of preventing hypomagnesaemic tetany. Proc. Brit. Soc. An. Prod. 1955, 109.

22. Effects of nitrogen and potassium fertilisers on the mineral status of perennial ryegrass. 1. Mineral content. J. Sci. Food Agric. 1960, 11, 422.

23. Effects of nitrogen and potassium fertilisers on the mineral status of perennial ryegrass. 2. Anion-cation relationships. J. Sci. Food. Agric. 1960, 11, 429.

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25. The effect of early weaning on the blood sugar and rumen acid levels of the growing calf. Brit. J. Nutr. 1964, 18, 29.

INTRODUCTION

A. The Chemistry of Ensilage

The major part of this thesis is concerned with the results of studies planned and carried out by the author during the past ten years on the chemical changes and losses occurring during the ensilage of herbage.

The object in initiating these studies was to examine systematically the various factors which influenced the ensilage process and to account chemically for the changes and losses which occurred. At the commencement of this work it was realised that in order to measure losses accurately an experimental silo unit in which a continuous record of weight changes could be made, was desirable. Consequently a unit, consisting of 4 metal silos, each with a capacity of 1000 kg fresh herbage, and individually suspended from a weighing device having a sensitivity of 1 in 10,000, was constructed[1,2]. In all, a total of 22 separate experiments have been completed using this unit, the majority of these being reported in this thesis[1,2,6,10,12,15,19,20].

Many of the existing methods of analysis were found to be unsuitable for silage and a number of new methods have been developed. These include in particular the determination of dry matter[3,4], water soluble carbohydrates and organic acids[13,14]. The conventional oven drying method of determining the dry matter content of foods is unsatisfactory for silages because of the losses of volatiles. A method based on the toluene distillation technique is described[4] and this method, which has been widely accepted as a standard procedure, has been used routinely in these studies. The water soluble carbohydrates (WSC) of grasses include glucose, fructose, sucrose, melibiose, stachyose and fructosans, and these are important as a source of readily fermentable carbohydrates for the lactic acid bacteria. A simple procedure for the determination of WSC in grasses and silages has been devised[11]. Where detailed information about the individual components of the sugar fraction was required chromatographic procedures were used[2]. The importance of hemicelluloses as a source of fermentable carbohydrates for lactic acid bacteria has also been examined[7].

During the course of these studies, the organic acids of plants assumed considerable importance because of their role in influencing the buffering properties of fermented herbage within the pH range 4-6. Considerable attention has therefore been devoted to the individual organic acids of grasses and clovers and the changes which they undergo during fermentation[13,14]. Clovers are particularly rich in organic acids; high concentrations of these are detrimental in achieving a satisfactory pH level and this fact is particularly important in ensiling legume crops[3].

Glyceric acid has been identified as the major acid present in both red and white clovers.

Considerable proteolysis occurs during ensilage[2], amino acids being the main end products in well preserved silage. Where clostridial activity occurs, however, decarboxylation of amino acids resulting in the formation of amines such as histamine and tyramine takes place[18]. These amines, if absorbed, have toxic effects and it has been postulated that histamine, in particular may be responsible for low intakes of dry matter associated with silages of high moisture content. The oral administration of histamine, as the dihydrochloride, in levels up to 1g/day to sheep on silage diets did not, however, appear to have any toxic effects nor adversely affect the animals' appetite[8]. It is concluded that this amine, if formed during ensilage, is normally broken down in the rumen.

In addition to the levels of soluble carbohydrate and organic acids in the crop, the following factors also influence the chemical changes and losses of nutrients during ensilage:- degree of consolidation, temperature, species and physical state of the crop, moisture content and bacterial population. The degree of consolidation influences the extent of anaerobiosis and affects the effluent production[2]. Consolidation is, however, simply a means of preventing re-entry of oxygen into the silage mass and with an adequately sealed silo is of secondary importance. The relationship between level of consolidation and extent of gaseous loss during ensilage has, however, been examined.

Considerable importance is attached to the influence of temperature on fermentation and the effects of this on biochemical changes and losses have been examined using thermo-electrically insulated silos[15]. The results confirm that high temperatures in silage are undesirable as they are associated with high losses of nutrients in the form of carbon dioxide resulting from aerobic respiration.

A number of different species of grasses and clovers have been examined in these studies and it is clear that there are variations in the ensiling properties of crops. Reference has already been made to the buffering capacity[5,14], in addition to this property the WSC content is of considerable importance. In order to obtain a satisfactory level of lactic acid for the preservation of unwilted grass silages, a minimum level of 8-10% WSC in the dry matter is considered desirable. Italian ryegrass (Lolium multiflorum) is usually rich in WSC and in an examination of over 50 different samples grown in S.E. Scotland, the lowest level recorded was 11%, and levels of over 20% WSC are commonly found. Cocksfoot (Dactylis glomerata) however, is frequently low in WSC content and levels of below 5% have been reported[6].

Apart from species and varietal variations, such environmental factors as season, climate, fertilisers and management can markedly influence their content in grasses. The effects of climate, fertilisers and season have been examined on ryegrass samples harvested from plots, together with a study of diurnal and quotidian variations. The results of these investigations, which have been reported in 'Experimental Work' 1963-67, confirm that the application of nitrogenous fertilisers, dull cloudy weather and matutinal harvesting all tend to reduce the WSC content of grasses. A quotidian study of the WSC of perennial ryegrass over 24 days indicated a positive correlation ($r=+0.74$) between WSC and hours of sunshine during the preceding 34 hour period. A study of the diurnal variation at 2 hourly intervals showed a range in WSC content of perennial ryegrass from 16.3% (8 a.m.) to 23.4% (4 p.m.). Clearly, where fermentable carbohydrates are critical as they may frequently be with cocksfoot and timothy/meadow fescue swards, a consideration of these factors may have an important practical application in silage making.

The moisture content of the crop is of major importance in influencing the pattern of fermentation and the losses of nutrients which occur. A clostridial fermentation is likely to develop when crops of high moisture content are ensiled and the importance of wilting herbage, preferably to a dry matter level of at least 30% has been demonstrated. Apart from discouraging a clostridial fermentation, the overall breakdown of nutrients is considerably reduced when wilted crops are ensiled[20].

Finally, a study of the ensilage process would be incomplete without a knowledge of the activities of the silage bacteria. In this respect the author is indebted to those members of the Bacteriology Department who gave him their co-operation. It has been demonstrated that fermentation by lactic acid bacteria is an extremely efficient means of conserving herbage. The losses of dry matter resulting from a heterolactic fermentation have been shown by both biochemical calculations and practical experiments to be of the order of 3-6%[19]. Where high losses occur during ensilage then these do not result from fermentation but are caused mainly by oxidation. The losses of dry matter occurring in the production of waste material for example, can be of the order of 75%[19].

The main conclusions and practical applications of these silage studies are outlined in three of the final papers[17,18,19] of the first section of this thesis.

B. Mineral and Digestibility Studies

The second part of this thesis is concerned mainly with mineral studies which were designed to study some of the factors concerned in the aetiology of hypomagnesaemic tetany. This section includes the results of a study of the effects of nitrogenous and potassic fertilisers on the mineral content and cation/anion ratios in perennial ryegrass.

C. Other Published Work

The author of this thesis is also the senior author of the textbook 'Animal Nutrition' by McDonald, Edwards and Greenhalgh, published in 1966 by Oliver and Boyd, Edinburgh. The author was responsible for the format of the book, the Appendix and Chapters 1,2,4,5,6,8,16,17,18, 19,20, 22.

ACKNOWLEDGEMENTS

The author would like to record his thanks to Professor S.J.Watson who initiated his interest in this subject. He would also like to thank the students and members of his staff who assisted in these investigations, and also members of the Microbiology department for many helpful discussions. Finally the author is indebted to the Agricultural Research Council for their help in providing a grant towards the cost of these studies.

SECTION A. THE CHEMISTRY OF ENSILAGE

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AN EXPERIMENTAL SILO UNIT FOR THE CONTINUOUS MEASUREMENT OF LOSSES OCCURRING DURING ENSILAGE

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An experimental silo unit consisting of four silos, each capable of holding 1000 kg. fresh grass, is described. The silos are suspended from a weighing apparatus enabling weight recordings to be taken at all stages during ensilage. The weighing apparatus is sufficiently sensitive to record a change in weight in the silo and contents of 100 grams.

INTRODUCTION

The accurate measurement of losses is of great importance in studying the biochemical and biological changes which occur during ensilage, and numerous experiments have been carried out using a wide range of silos. Watson (7) has reviewed in detail many of these experiments and more recently Allred *et al.* (1) and Larrabee and Sprague (4) have discussed the extent of effluent, spoilage and fermentation losses under different conditions.

It is evident that a large variation in nutrient losses can occur during ensilage. However, the figures for the losses reported are often complicated by the fact that some workers include spoiled silage in their calculations and unless these losses are specified a false impression of the efficiency of the ensilage method of conservation may be obtained, since spoilage losses are associated with the mechanics of the process.

The difficulties in studying changes occurring in large farm silos have been stressed by Barnett and Miller (2). These authors concluded that where comparative results are needed, the use of small-scale silos is essential. A large number of experiments have been carried out by different workers using silos of this type in which the balance of nutrients remaining at the end of the preservation period has been calculated. The majority of these workers agree that unless care is taken, the errors involved in weighing fresh material, in sampling and in analytical techniques can be considerable and that the interpretation of the results in terms of losses caused by a wide variety of factors of chemical, biological and mechanical origin is very difficult. Most of the investigations already carried out provide little information about the rate of loss during ensilage since the final fresh weights of the silages were determined after the silos had been emptied. The rate of loss can be important in studying the effects of different treatments upon ensilage and a system where the changes in weight from day to day can be recorded provides information on the loss at any given stage of the process and eliminates the necessity for transferring silage from the silo into containers for weighing. Kroulik *et al.* (3) have already described small (4 ft. × 8 ft.) steel silos which were transportable for weighing. A less laborious system is to incorporate the silos with a weighing device. A silo unit consisting of four metal silos, each having a capacity of 1000 kg. fresh grass and each suspended from weighing apparatus, has been constructed at the Bush Estate, Edinburgh, and is described below.

CONSTRUCTION OF SILOS

The silos (Plate I and II), housed indoors and constructed of $\frac{3}{16}$ in. mild steel, are 5 ft. (153 cm.) diameter × 6 ft. (183 cm.) high, containing a 2 × $\frac{3}{8}$ in. strengthening flange round the top. The bottom of each silo, which is reinforced with a strong ring of steel, slopes gently to a central outlet of $4\frac{1}{2}$ in. diameter fitted with a gun-metal screw-cap containing a short drainage pipe of $\frac{1}{2}$ in. diameter. The insides of the silos are coated with bitumen.

Each silo is supplied with seven sampling ports of 4½ in. diameter fitted with airtight screw caps of similar material to the effluent cap. These ports are arranged at different levels round one third of the circumference of the silo, the vertical distance between each port being 9 in. The inside of the silo is graduated in centimetres so that the volume occupied by the herbage can be calculated at any given time.

Each silo is suspended by means of three chains, each attached to a 1 in. steel tie-rod so that the lift is taken from the base of the silo. Final consolidation of the grass can be regulated by placing stone blocks on top of a wooden compression disc which fits closely inside the silo. This disc is made in two sections for ease of handling.

For effluent collection, a plastic tube is attached to the short drainage pipe and is led into a 10-litre polythene container, which is suspended from the front side of the silo. The plastic tube is fitted with a glass tap which is closed when the effluent is being removed.

In each silo there are nine thermocouples leading to a single recording unit. Individual temperature readings can be taken when required.

WEIGHING APPARATUS

The silos are suspended from the weighing apparatus which is bolted to a 20 ft.-long steel **I** beam (10 × 5 in.), supported on three brick pillars. Each beam supports two silos. The two outer pillars, which are 1½ ft. square and 10 ft. high, rest on 1 ft.-deep concrete bases 3½ ft. square. The stronger central pillar of each unit is 1 ft. 10½ in. square with a concrete base 3 ft. 10½ in. square.

During filling, each silo rests on 6 concrete blocks insulated with ¼ in. rubber sheeting and covered with removable wooden wedges (not shown in Plates 1 and 2). One of the blocks can be removed during experiments to simplify effluent collection.

The weighing apparatus is of the steelyard type and is designed on the principle of counterbalancing the whole load and measuring the actual weight loss, similar to the method described by Robertson (6) for tripod-hay weighing. The steelyard is constructed of a 6 in. × 4 in. rolled steel joist, 5 ft. in length, which permits a maximum mechanical advantage of 9 : 1.

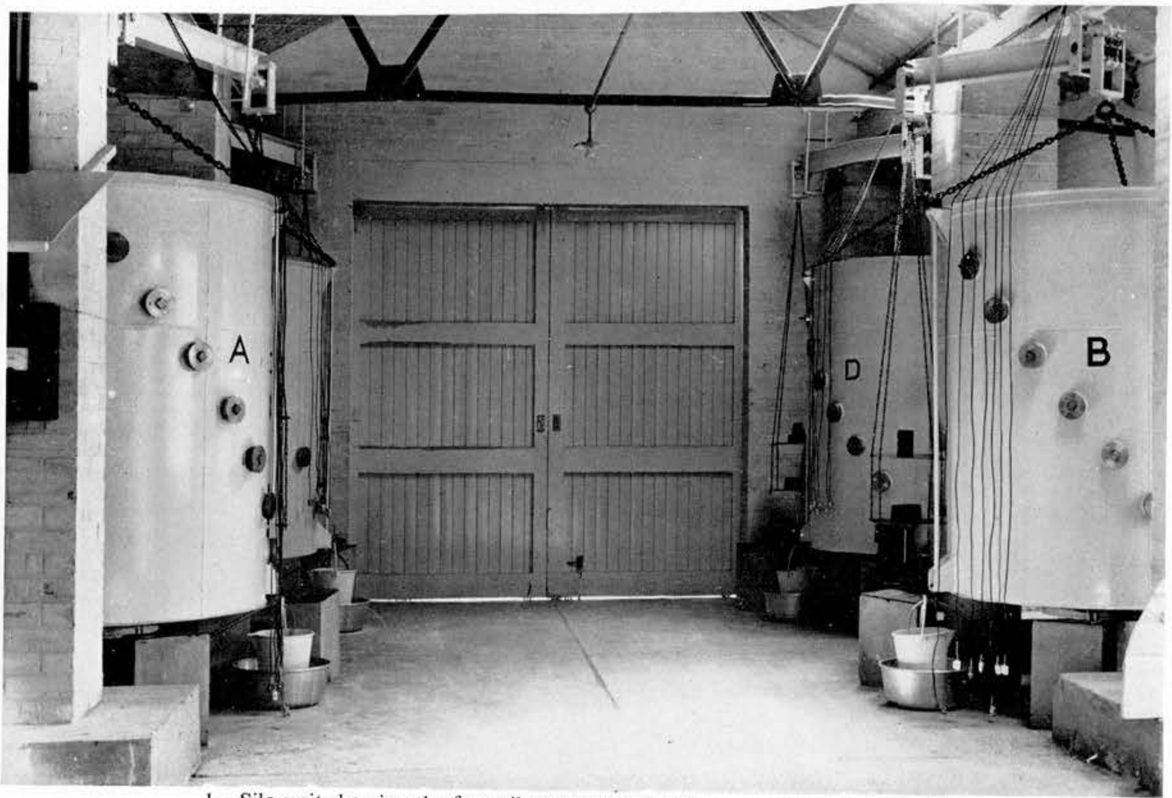
The cradles supporting the silo and weight-pan contain case-hardened knife-edges machined to very close limits. The central supporting cradle is not knife-edged but contains a ball-race with the minimum number of balls necessary to carry the maximum load.

In this system it is important that the weighing beam is close to the horizontal position during operation, otherwise the centre of gravity moves slightly and reduces the mechanical advantage. The loss in weight is recorded on a spring balance which is under tension. Its vertical position is adjustable to simplify recording.

The mechanical advantage of the prototype (silo C) was determined by using known weights in the silo and found to be 8·62. The mechanical advantage was increased slightly in constructing the other three weighing units by improving the design of the suspension bearings.

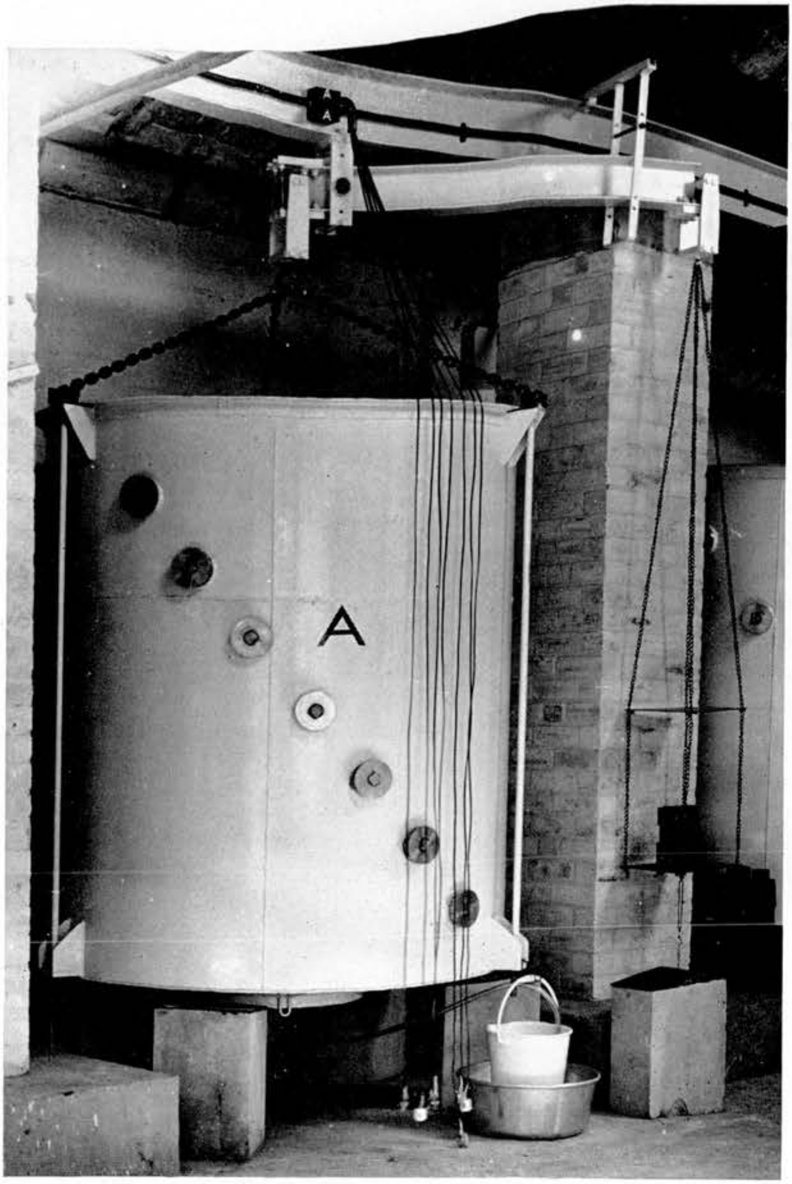
EXPERIMENTAL METHOD

An experiment was carried out in the autumn of 1956 to determine the efficiency of the silo unit. At this time only one of the silos (C) had been set up. The grass ensiled was at a mature, stemmy stage of growth with the seed heads fully developed and was obtained from a second-year perennial ryegrass/cocksfoot/white clover ley. The dry-matter content of the grass was 23·65% and the nitrogen content, on a moisture-free basis, 2·39%. The silo was filled on 12 October 1956. During filling, the grass was consolidated and finally covered with a layer of polythene sheeting. The wooden compression disc was placed on top of the sheeting and weighted with stone blocks. The weight of the latter (including disc and sheeting) was 353 kg., equivalent to a pressure of about 20 g./sq. cm. The quantity of grass ensiled in this experiment was 373·8 kg. The effluent outlet was sealed during the experiment and the losses recorded were therefore due solely to gaseous products of fermentation and respiration. Since construction of the thermocouple



1. Silo unit showing the four silos suspended with temperature leads outside.

PLATE I



2. Single silo and weighing apparatus.

PLATE II

unit had not been completed at the beginning of the experiment, no temperatures could be recorded.

The silo was opened on 21 February 1957 and a representative sample taken for analysis. The sampling and analytical technique were as described in an earlier paper (McDonald and Purves 5).

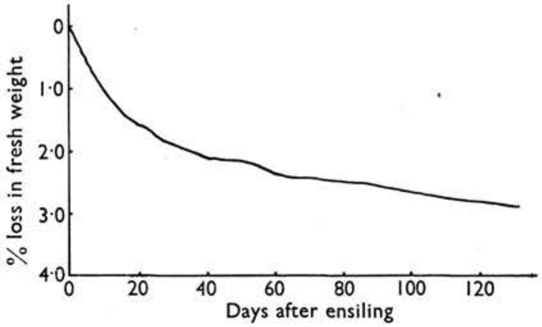


Fig. 1. Rate of gaseous loss expressed as percentage of fresh weight of grass ensiled.

RESULTS

The weight changes were measured daily and the gaseous losses are shown in Fig. 1. The final weight of silage recorded on the apparatus (361·9 kg.) was carefully verified on an accurate weighbridge. It was found during weighing that the sensitivity of the apparatus was such that a change in weight in the silo of 100 g. could be recorded. This corresponds in this experiment to a sensitivity of approximately 1 in 10,000.

The silage had a pH value of 5·7 and the dry-matter content, corrected for volatile acids and nitrogen lost during drying, was 19·5%. The nitrogen content on a moisture-free basis was 2·95%. The losses during ensilage in dry matter and nitrogen were 19·97 and 1·44%, respectively.

DISCUSSION

The main purpose of this first experiment was to determine the accuracy and reliability of the weighing apparatus. The mechanical advantage had been determined prior to filling at different weight levels and was found to be constant. The sensitivity of the apparatus was also high and small changes in weight could be detected. The greatest difficulty in designing the weighing equipment itself was the accurate measurement of small changes in the silage mass. The silo weighed 560 kg. and when completely filled with herbage and consolidated could give a maximum load of about 2500 kg. Direct weighing, using electrical-resistance strain-gauges or pressure-type load-cells, was considered but found to be impracticable and because of this the steelyard type of weighing apparatus was used. The design of the equipment necessitated the reduction of friction to a minimum and although a multi-beam steelyard would have been satisfactory, this could not be accommodated in the space available and it was therefore necessary to use a single steelyard sensitive to small fluctuations in weight.

The sensitivity of the equipment has been verified in a further experiment in which practically identical well-preserved silages were produced in all four silos from a cut of pure Italian ryegrass. The detailed results of this experiment will be reported in a later paper.

ACKNOWLEDGMENTS

The authors would like to thank Prof. S. J. Watson and Dr. A. M. Smith for their encouragement, Mr. C. H. C. Mathews for the construction of the temperature equipment, Dr. M. J. Nash, Mr. A. Hardie, Miss A. Henderson and Mr. G. Davie for advice and practical assistance. The construction of the unit was made possible by a grant from the Agricultural Research Council to the University of Edinburgh.

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STUDIES ON ENSILAGE

EDINBURGH SCHOOL OF AGRICULTURE
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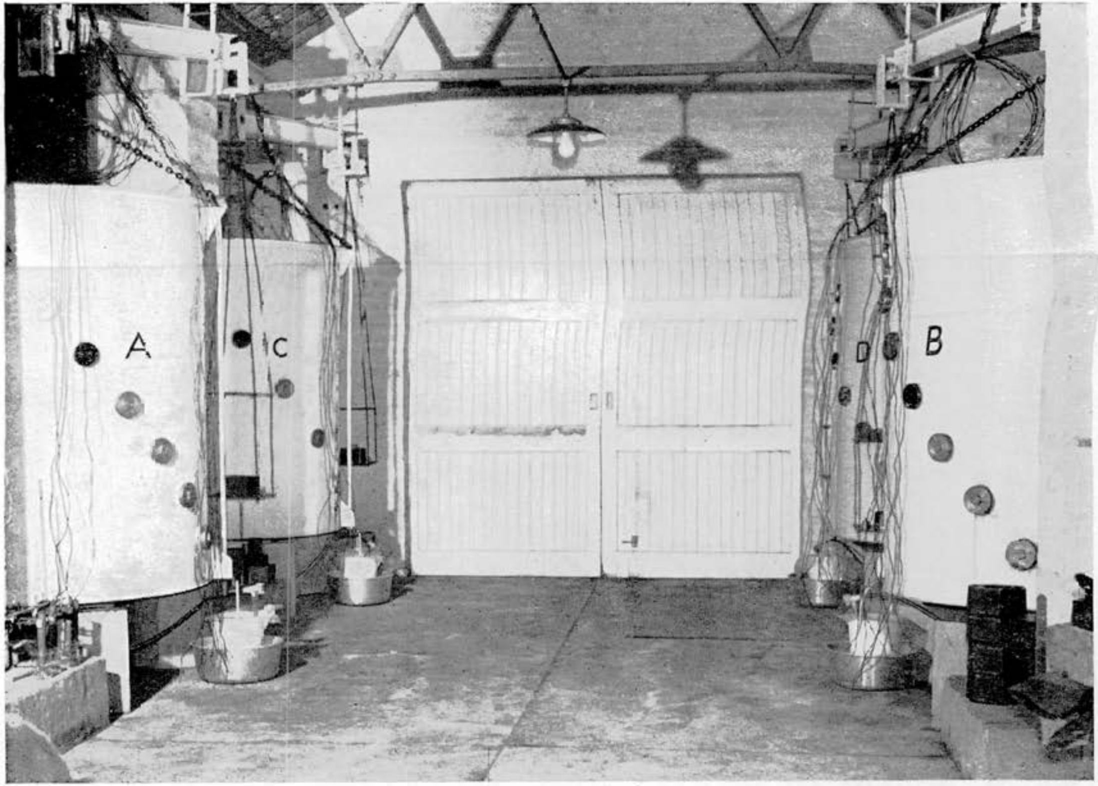

Plate 1. Silo Unit.

THE EDINBURGH SCHOOL OF AGRICULTURE

STUDIES ON ENSILAGE

(i) Construction of experimental silos

(ii) Techniques

(iii) The effect of consolidation

By

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1960

FOREWORD

Much of the work on the conservation of green crops for winter use is of an experimental nature without adequate replication or suitable control. The variables are often so many that the results are quite incapable of proper interpretation. It was obvious that some understanding of the nature of the processes involved was essential if proper advice was to be given to the agricultural industry.

For the last ten years the Edinburgh School of Agriculture and the Department of Organic Chemistry of Edinburgh University have been working on the fundamental aspects of the conservation of grass. This was made possible by a very generous grant to the University by the Agricultural Research Council over the period in question.

The work is still continuing, though on a reduced scale, but it is clear that this line of attack on the problems involved in the conservation of forage crops is the only one likely to yield results of importance.

This publication, which puts on record the results obtained by a number of workers over the last few years illustrates the complexity of the problem. It also summarises the results obtained with a battery of silos specially erected for the work on funds provided by the Agricultural Research Council. This apparatus has already furnished some very valuable information and is in constant use.

The references also indicate the progress made in this fundamental work, many of them being by members of the team working at Edinburgh on the problems of conservation and obtained in the course of the work described. Chemists, bacteriologists and agriculturists have all co-operated and this work still continues. This is the first of the detailed accounts that it is hoped to present of the work carried out by the co-operative effort of these workers at Edinburgh. Its publication is only possible as a result of a special grant from the Agricultural Research Council to which we are grateful for all the assistance so readily given during the course of the investigations.

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CONTENTS

SUMMARY

THE construction of an experimental silo unit for the continuous measurement of losses during ensilage has been described. The silo unit consisted of four metal silos, each having a maximum capacity of 1,000 kg. fresh herbage.

A detailed description has been given of techniques used in chemical, bacteriological and nutritional studies on herbage and silage. These include the measurement of individual carbohydrates by chromatographic separation, nitrogenous compounds, lactic and volatile acids and dry matter determinations. Bacteriological examinations were carried out on the fresh herbage and silage made from it. The bacterial development was also followed in the laboratory using small test tube silos. The nutritional investigations included digestibility determinations and nitrogen utilisation measurements using sheep.

The purpose of Experiment 1 was to assess the degree of variation which could occur during ensilage in the four silos filled under identical conditions with Italian ryegrass.

In general similar changes occurred in all four silos during ensilage. The day to day changes as measured by weight and volume recordings and effluent analysis also showed a similar pattern.

The resultant silages were well preserved (pH 3·7-3·9) and the losses in dry matter were of a low order (6·6-8·2 per cent). Lactobacilli became dominant within the first three days in the laboratory silages and remained dominant throughout the experiment. Lactobacilli were also the dominant organisms in the sample taken from the large silos. The nutritional values of the grass and silages were similar.

The purpose of the second and third experiments was to study the effect of variations in consolidation within the silo. In the second experiment, although well preserved silages were produced in the four silos, the amount of top spoilage was greatest in the less consolidated silos.

In the third experiment an attempt was made to produce high temperature silage. The maximum temperature obtained was only 44°C, in spite of poor compression during ensilage.

The digestibility of the silage produced at this temperature was inferior to that of silages made at lower temperatures. The significance of this is discussed.

In all three experiments silo A was always filled under identical conditions which made possible a direct comparison of the fermentation reactions. A comparison between summer and autumn grass of similar chemical composition showed that resultant silages were dissimilar with higher losses occurring in ensiled autumn grass than in the summer herbage. The importance of the bacterial flora on the original herbage in connection with these losses has been discussed.

INTRODUCTION

THE accurate measurement of losses is of great importance in studying the biochemical and biological changes which occur during ensilage, and numerous experiments have been carried out using a wide range of silos.

It is generally agreed that in order to carry out detailed studies during ensilage, the use of small scale silos is essential. The use of small silos for experimental studies is not new and as early as 1866 March, described by Potter (1), produced iron cylindrical silos capable of holding from 3 cwt. up to 20 cwt. of silage. Since this time, experimental silos ranging in size from test tubes to large towers holding several tons of herbage have been constructed. Watson (2) has reviewed in detail many of these experiments. More recently Barnett (3) has experimented with laboratory silos and Perkins, Pratt and Rogers (4) have described small cylindrical silos made of glass lined steel and of nine pounds capacity, while Nilsson Tóth and Rydin (5) have carried out extensive experiments using small scale silos of stainless steel holding 12-15 kg. of fresh herbage. Slightly larger silos made of glass were used in silage studies by Axelsson and Eriksson (6) and Foot, Murdoch and Rowland (7) have carried out experiments with silos of 15 ton capacity fitted with thermocouples.

Unless care is taken, the errors involved in weighing fresh material, in sampling and in analytical techniques can be considerable and the interpretation of the results in terms of losses caused by a wide variety of factors of chemical, biological and mechanical origin, is very difficult. Most of the investigations already carried out provide little information about the rate of loss during ensilage since the final fresh weights of the silages were determined after the silos had been emptied. The rate of loss can be important in studying the effects of different treatments upon ensilage and a system where the changes in weight from day to day can be recorded provides information on the loss at any given stage of the process and eliminates the necessity for transferring silage from the silo into containers for weighing. Kroulik *et al.* (8) have already described small (4 ft. × 8 ft.) steel silos which were transportable for weighing. A less laborious system is to equip the silos with a weighing device. In the experiments described in this paper a silo unit consisting of four metal silos, each having a capacity of 1,000 kg. fresh grass and each suspended from weighing apparatus has been used. The construction of this unit and techniques used in the silage experiments are described in detail below.

CONSTRUCTION OF SILO UNIT

Silos

The silos (Plate 1) are housed indoors and are constructed of mild steel, 5 ft. (153 cm.) diameter $\times$ 6 ft. (183 cm.) high, containing a strengthening flange round the top. The bottom of each silo, which is reinforced with a strong ring of steel, slopes gently to a central outlet of $4\frac{1}{2}$ in. diameter fitted with a gunmetal screw cap containing a short drainage pipe of $\frac{1}{2}$ in. diameter. The insides of the silos are coated with bitumen.

Each silo is supplied with seven sampling ports of $4\frac{1}{2}$ in. diameter fitted with airtight screw caps of similar material to the effluent cap. These ports are arranged at different levels round one-third of the circumference of the silo, the vertical distance between each port being 9 in. The inside of the silo is graduated in cm. so that the volume occupied by the herbage can be calculated at any given time.

Each silo is suspended by means of three chains each attached to a steel tie rod so that the lift is taken from the base of the silo. Final consolidation of the grass can be regulated by placing stone blocks on top of a wooden compression disc which fits closely inside the silo. This compression disc is made in two sections for ease of handling.

For effluent collection, a plastic tube is attached to the short drainage pipe and is led into a 10 litre polythene container, which is suspended from the front side of the silo. The plastic tube is fitted with a polythene tap which is closed when the effluent is being removed.

In each silo there are ten thermocouples leading to a single recording unit. Individual temperature readings can be taken when required.

Weighing apparatus

The silos are suspended from the weighing apparatus which is bolted to a 20 ft. long steel I beam (10 in. $\times$ 5 in.), supported on three brick pillars. Each beam supports two silos. The two outer pillars rest on deep concrete bases.

During filling each silo rests on 6 concrete blocks insulated with rubber sheeting and covered with removable wooden wedges (not shown in Plate 1). One of the blocks can be removed during experiments to simplify effluent collection.

The weighing apparatus is of the steelyard type and is designed on the principle of counterbalancing the whole load and measuring the actual weight loss, similar to the method described by Robertson (9) for tripod hay weighing. The steelyard permits a maximum mechanical advantage of 9 : 1.

The cradles supporting the silo and weight-pan contain case-hardened knife-edges machined to very close limits. The central supporting cradle is not knife-edged but contains a ballrace with a minimum number of balls necessary to carry the maximum load.

In this system it is important that the weighing beam is close to the horizontal position during operation otherwise the centre of gravity moves slightly and reduces the mechanical advantage. The loss in weight is recorded on a spring balance which is under tension. Its vertical position is adjustable to simplify recording.

The mechanical advantage of the prototype (silo C) was determined by using known weights in the silo and found to be 8·62. The mechanical advantage was increased slightly in constructing the other three weighing units by improving the design of the suspension bearings.

Sensitivity

The sensitivity of the apparatus is such that a change in weight in the silo of 100 g. can be recorded. This corresponds, when maximum loading is applied, to a sensitivity of approximately 1 in 25,000. The accuracy of the equipment was verified in a preliminary experiment. Details of this and the construction of the equipment have been given by McDonald and Attwood (10).

TECHNIQUES

(A) Sampling

Accurate sampling of plant material from a large bulk is difficult, especially when samples have to be removed without disturbing the mass.

With silage *in situ* considerable variations can exist in both horizontal and vertical directions owing to the effects of consolidation and aeration during fermentation. Horizontal cores taken at different levels tend to overcome these differences, although mechanical pressure during the coring operation frequently results in samples of higher dry matter values being obtained. Because of this it is unwise to attach too much importance to 'balance' calculations based on analytical results from cored samples. The latter, however, can be a useful guide to changes occurring during ensilage, although it is essential to avoid secondary fermentation reactions occurring due to the introduction of oxygen during sampling. Hence the practice in our studies where coring has been employed has been to inject nitrogen into the core hole prior to sealing with the port cap.

In the present experiments, calculations of losses were based on representative samples taken by hand sampling from herbage going into the silo and from silage removed at the end of the experiment. During the sampling operation polythene gloves were worn. Sufficient hand samples were taken to represent a total of about 2 per cent of the weight of herbage or silage material in each silo. The hand samples were stored in a large polythene bag until the filling or emptying operation was complete, and were then carefully mixed and sub-sampled by 'quartering.' The small sub-sample was cut into small lengths by hand-shears. This method has been found preferable to machine mincing because mincing squeezes out moisture and may also heat the sample.

(B) Analysis of grass, silage and effluent samples

(i) *Dry matter and acids*

(*a*) *Fresh grass.*—The grass was dried for 24 hours at 100°C in a forced air electric oven. The dried samples were hammer milled through a 1 mm. mesh sieve and stored in screw capped bottles until analysed.

(*b*) *Silage*.—The volatile fatty acids in the fresh silage were determined by Wiseman and Irvin's method (11). The lactic acid was determined by the ceric sulphate oxidation method of Elsden and Gibson (12). Details of these methods are given in Appendix II.

The determination of dry matter of silage presents difficulties because of the losses of volatile constituents during the normal drying process. Watson and Ferguson (13) have discussed the importance of considering these volatile losses and have suggested correcting the apparent dry matter values by estimating the volatile bases and volatile acids in the fresh and dried samples. The method adopted in this work involved the drying of 100 g. fresh silage in a flask which was thermostatically heated at 100°C and through which a current of dry air was passed. The moisture and volatile constituents were condensed and collected in a weighed flask at $-$ 10°C. Determinations of volatile acids, lactic acid, and ammonia were made on the distillate and, by subtraction, the true moisture content and hence the dry matter of the silage was calculated. Full details of this method are given in Appendix II.

(*c*) *Effluents*.—The dry matter of the effluent was determined by correcting the 'apparent dry matter' value, obtained by drying a 50 ml. sample overnight in an electric oven maintained at 100°C, for volatile acids and volatile nitrogen (calculated as NH_3). The procedure is given in detail in Appendix II.

(ii) *Routine*

Crude protein (C.P.), ether extract (E.E.), crude fibre (C.F.), nitrogen-free extractives (N.F.E.) and ash were determined by the methods laid down in the Regulations of the Fertilisers and Feeding Stuffs Act (14). These determinations were carried out on the dried milled samples. In the case of silages, corrections were applied to the percentage figures in order to allow for the volatile materials lost during drying.

Since the amounts of volatile acids lost during drying would be included in an ether extract from fresh silage, it is common practice (Watson, 2; Brown and Heaney, 15) to correct the values for ether-extractable material obtained from dried silage for volatile acids lost. Such corrections have not been applied in this work for a number of reasons. The bulk of the volatile acids in silage may be considered as being derived from carbohydrates included in the nitrogen-free extractives fraction; therefore the addition of the volatile acid lost on drying to the ether-extractable material involves a transference of material to a different fraction, complicating the problem of assessing losses of individual constituents in the silo as well as falsifying the starch equivalent and total digestible nutrients results. The volatile acids lost on drying have therefore been included in the values for nitrogen-free extractives.

(iii) *Carbohydrates*

Grass carbohydrates may be divided into two types :—

The non-structural or water-soluble components ;
The structural components.

The water-soluble carbohydrates may be sub-divided as follows :—

(*a*) Free sugars—D-glucose, D-fructose and sucrose ;
(*b*) Oligosaccharides—melibiose, raffinose, stachyose and short chain fructosans ;
(*c*) Fructosan—This sugar polymer appears to be present in ryegrasses and is absent from the clover species.

The structural carbohydrates are :—

(*a*) Cellulose—estimated as glucosan ;
(*b*) Hemicelluloses—comprising pentosans and galactan.

Lignin because of its association with the structural carbohydrates has also been determined in these studies.

Samples of grass or silage for analysis were taken as previously described and after weighing were immediately immersed in boiling ethanol to inhibit enzymic action. A scheme for the analysis of the carbohydrate material in grass and silage based on the work of Wylam (16) and Harwood (17) has been evolved.

Firstly, it was necessary to divide the carbohydrate material, by successive treatments with different solvents, into suitable fractions. The raw material was extracted with 80 per cent ethanol in a Soxhlet apparatus to give a fraction containing the free sugars and oligosaccharides, then with cold water to obtain the fructosan fraction. The residue was treated with boiling NH_2SO_4 to extract and hydrolyse the easily accessible polysaccharides. Subsequent treatment with 72 per cent H_2SO_4, at room temperature, hydrolysed the cellulose, leaving a residue which was principally lignin.

In the case of these complex carbohydrates, where hydrolysis to component sugars was necessary before estimations could be undertaken, the results were expressed as xylan, araban, galactan and glucosan. This does not necessarily imply that any sugar was combined solely with itself as a polymer. Further these carbohydrate fractions had to be freed from non-carbohydrate material. This was of major importance only in the alcohol fraction, as most of the extraneous matter, such as chlorophyll, accumulated in this fraction. The removal of such matter by 'clarification' was carried out by the addition at 90°C of suitable volumes of $CdSO_4$ and $Ba(OH)_2$. 'Deionisation' was then carried out to remove any inorganic ions introduced by the clarification procedure. The method used was that of ion-exchange electrodialysis using 'Permaplex C–20' and 'A–20' membranes as described by Anderson and Wylam (18).

The sugars in each case were separated chromatographically and, after hot water elution, were estimated directly by means of the Somogyi method where possible. The sucrose and the oligosaccharides were hydrolysed to component sugars before estimation. The fructosan was estimated by Roe's colorimetric method as used by Wylam (16).

The sugars in the effluent were determined by the Somogyi method on the clarified effluent before and after hydrolysis for four hours with 0·5 NH_2SO_4.

(iv) *Nitrogenous compounds*

The grass and silages were analysed for total nitrogen, non-protein nitrogen and volatile nitrogen. The analysis was carried out on samples of fresh material to avoid proteolysis in fresh grass and loss of volatile base in silages, which occur on drying. Determinations of total amide nitrogen ($CONH_2$-N), glutamine, and asparagine amide nitrogen in the effluents were also carried out. Full details of the methods of analysis are given in Appendix II.

(v) *Gas analysis*

Gases were not analysed in these studies and the calculated gaseous losses refer to total losses less effluent and might be an underestimate if the residual gases were mainly carbon dioxide.

(C) Metabolism studies

Digestibility trials and nitrogen balance studies were carried out on grass and silages during these experiments. The equipment used in these metabolism studies for collection of faeces and urine was similar to that described by McDonald (19).

The digestibility trials consisted of a preliminary feeding period of seven days on the experimental diet. For two weeks prior to this the sheep had been kept on a grass or silage diet in order to avoid any sudden change in the ration. The experimental feeding period lasted thirteen days and consisted of two five-day sub-periods during which daily samples of feed were taken. These were dried separately then bulked in five-day lots for subsequent analysis. Any uneaten residues in the feeding boxes were weighed and, if different in appearance from the original feed, analysed.

Faeces were also collected over a ten-day period, there being a three-day time-lag between commencement of feeding and the first collection of faeces. The daily faecal collection from each sheep was weighed and, after thorough mixing, a 20 per cent aliquot sample to which a few drops of chloroform had been added was kept at 0-4°C. These aliquot samples were bulked in five-day sub-periods, thoroughly mixed, and analysed for nitrogen. When losses of nitrogen occurred during drying, the dry matter values were corrected, calculating the volatile nitrogen as NH_3.

When digestibility studies on the fresh grass were carried out, samples of the herbage were taken from material which was used for filling the silos, stored in polythene bags (30 in. $\times$ 15 in.) and kept in a deep freeze refrigerator about $-$ 10°C. This frozen grass was collected daily from the deep freeze unit over a fourteen-day period and, after thawing at room temperature for about an hour, fed to the sheep.

Samples of silage were transferred to cylindrical metal bins (76 cm. $\times$ 35 cm.), lined with polythene, to which a few pieces of solid CO_2 had been added to displace the air. The silage was consolidated in the bins which were then sealed with tight-fitting lids. The material was satisfactorily conserved in this way during the period of the digestibility trials. The silage trials took the form of a reversal experiment which enabled four treatments to be replicated three times with six animals.

(D) Bacteriological investigations

In addition to an examination of the bacterial populations of the fresh herbage and of the silage made in the experimental silos, the development of bacteria was followed by examining at intervals silage made under controlled conditions in the laboratory. For the bacteriological investigations small handfuls of the fresh herbage abstracted during the filling of the silos were accumulated in a sterile bag for transportation to the laboratory. The worker who was sampling wore sterile rubber gloves. At no time during subsequent manipulations was the grass allowed to come in contact with unsterile surfaces.

In the laboratory the grass sample was well mixed and then 50 g. quantities were filled into glass tubes (c. 20 cm. $\times$ 3 cm.). The grass was packed as evenly as possible by means of a wooden rod of diameter slightly less than that of the tube. Each tube was closed with a rubber stopper carrying a valve of mercury over sintered glass which permitted the escape of gases but prevented the entry of air. The filled tube 'silos' were placed in the dark in a thermostatically controlled water-bath.

A further 50 g. of the grass was placed in a tared macerator jar, sterile water was added until the suspension weighed 300 g., and the material was then disintegrated for 2 min. by an electric top-drive macerator. The macerate was used for bacteriological examination and for the electrometric determination of pH. Appropriate decimal dilutions of the macerate were plated on a variety of media designed to give counts of groups of silage organisms, *e.g.*, the lactic acid bacteria, Gram-negative bacteria and anaerobic bacteria.

After varying periods of time tube silages were examined in the same manner, the whole content of a tube constituting the sample for each examination.

When the large experimental silos were emptied, the silage was sampled in the same way as for the fresh herbage. Small quantities were taken at intervals ensuring the representation of material from the top to the bottom of the silo. Obvious waste material was collected separately. The sample from each silo was well mixed in the laboratory, a sub-sample was cut into short lengths and again mixed, after which 50 g. were macerated in the manner described above.

The bacteriological examinations thus covered (*a*) the fresh herbage, (*b*) the silage removed from each experimental silo, and (*c*) stages during the fermentation of the same herbage under conditions of laboratory control.

Details of the media used and of the methods of examination are given in Appendix II.

EXPERIMENT 1

Experimental

THE purpose of this experiment was to examine the consistency of results from the four silos under identical conditions of ensiling. The grass used was from a pure sward of Italian ryegrass (Lolium italicum S22) which had been established the previous year. The grass was cut with a mowing machine on 5th July 1957, this being the third cut taken from the field during 1957. The silos were filled up to the 160 cm. level and the herbage was well consolidated by tramping during filling. The volume occupied by the fresh herbage was 3·02 cu. m. The silos with contents were weighed prior to covering the surface with polythene sheeting, wooden compression discs and stone blocks. The quantities of grass ensiled were 910, 906, 906 and 911 kg. in silos A, B, C and D respectively. The corresponding consolidation weights applied were 678, 683, 679 and 676 kg. which approximated to a pressure of 37 g./sq. cm.

The silos and contents were weighed daily. Effluents were collected daily, or when they appeared, and were analysed for dry matter (corrected), ash, nitrogenous constituents, sugars and volatile acids. Temperatures were not recorded since recording equipment had not been completed at the commencement of the experiment. The silos were opened on the 5th September 1957, *i.e.* sixty-three days after filling. A small amount of mould had developed in the top layers in all four silos and this material was discarded after weighing. The quantities of this 'spoiled' material are given below :—

	Weight of 'spoiled' material kg.	*% of original grass ensiled*
A	14·07	1·55
B	9·42	1·04
C	12·49	1·38
D	16·12	1·77

Results and Discussion

The quantities of grass ensiled and the weights applied were similar in the four silos. One factor of great importance in experiments of this kind is to ensure that the same degree of consolidation occurs in each silo. Consolidation can be regarded as a function of two factors : firstly, degree of compaction during filling, and, secondly, weight applied to the herbage after filling (compression). Both of these factors can be kept constant provided the same weight of material is ensiled in the same volume and similar weights are applied on top of the herbage after filling the silo. The relative density of the Italian ryegrass used in these experiments was 0·77 and from a knowledge of the volume of each silo (3·02 cu. m.) it can easily be calculated that the true volume occupied by the herbage was only 1·18 cu. m. and the difference of 1·84 cu. m. must have been air. It is known, however, that the density of minced grass is nearer unity than that of unchopped grass and it is obvious that a certain amount of gaseous material is always present in the plant tissues. Because of this, too much significance cannot be attached to density determinations. In these experiments, however, similar weights of grass were ensiled in similar volumes and since the consolidation weights applied after filling were identical it follows that the amount of consolidation in each silo was identical. Figure 1 shows the changes in volume occupied by the silage during the experiment; the rate of fall in the levels was similar in all four silos and it can be seen from the graph that the greatest change in volume occurred in the first five days of the experiment. During this period the volume fell to less than half of the original volume.

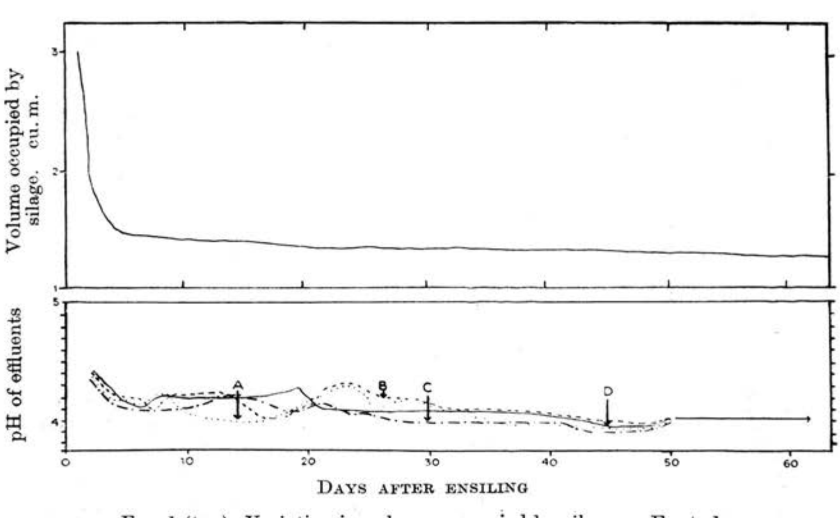

FIG. 1 (top). Variation in volumes occupied by silages. Expt. 1
FIG. 2. Variation in pH of effluents. Expt. 1

COMPOSITION

The chemical composition of the original grass and the four silages are shown in Table 1. It can be seen from the pH values and the acid contents that all four silages were in a

TABLE I.

EXPERIMENT 1

Composition of grass and silages (per cent dry matter)

	Grass	Silages			
		A	B	C	D
*Dry matter	19·16	19·17	19·18	19·10	18·83
incl. volatile acetic acid		0·32	0·33	0·33	0·31
„ „ lactic acid		0·15	0·16	0·15	0·17
„ „ ammonia		Nil	Nil	Nil	Nil
Organic matter	89·8	88·3	88·5	88·3	88·6
Crude protein	18·7	18·5	19·1	18·7	19·6
Ether extract	3·5	4·9	4·6	4·8	4·8
Crude fibre	23·6	26·2	26·1	25·7	25·9
N.F.E.	44·1	38·7	38·6	39·1	38·4
Total N	3·00	2·96	3·06	2·99	3·13
Protein N	2·66	0·88	0·98	0·91	1·05
Non-protein N	0·34	2·08	2·08	2·08	2·08
Volatile N		0·21	0·21	0·21	0·21
Total Carbohydrates incl. lignin	65·5	42·0	42·3	45·3	38·3
Total Sugars	9·5	2·2	1·6	2·0	1·5
Sucrose	3·5	0·1	tr.	0·1	tr.
Glucose	2·2	0·2	tr.	0·2	tr.
Fructose	1·9	tr.	tr.	tr.	tr.
†Oligosaccharides	2·0	0·4	tr.	0·4	0·5
Galactose		0·6	0·6	0·5	0·4
Arabinose		tr.	tr.	0·2	tr.
Xylose		0·9	0·9	0·7	0·5
Fructosan	5·6	0·3	0·3	0·2	0·3
Total Hemicelluloses	15·9	7·8	11·3	13·7	9·5
Galactan	1·1	0·3	0·8	1·1	0·6
Araban	3·0	0·8	1·0	2·6	1·4
Xylan	11·8	6·7	9·5	9·1	7·6
Cellulose (C. & M.)	24·9	26·6	27·0	26·8	27·1
Cellulose (glucosan)	26·2	26·0	23·6	23·3	21·3
Lignin	8·3	5·8	5·6	6·2	5·7
Lactic acid		8·7	9·5	8·7	8·4
Acetic acid		1·8	1·8	1·8	1·9
*pH		3·8	3·7	3·9	3·8

* pH and volatile constituents determined on fresh material.
† Excluding sucrose.

similar state of preservation. The dry matter content of the silage did not differ from that of the original grass to any extent, the range over the five results being within 2 per cent. If the dry matter values of the silages had not been corrected for volatile losses on drying, they would have been 2·5 per cent lower than reported. In silo A this error would have increased the dry matter losses from 7·02 to 9·26, *i.e.* an increase of over 30 per cent. The importance of accurate dry matter determinations is obvious in balance experiments of this type.

Although there is little difference in total N content between the original grass and silages, considerable change in the distribution of the N compounds had occurred. A similar alteration in carbohydrate constituents had occurred and here some differences between the four silages existed. It is doubtful if much significance can be attached to the variation in total sugar values because of the small quantity of these substances present in silages compared with the original grass. The main sugars present in the alcohol extracts from the silages were xylose and galactose, these being absent from the original ryegrass; these two sugars along with arabinose must have come from a breakdown of hemicelluloses during ensilage. It is interesting to note the extremely low values for fructosan in the silages. It is customary to regard the total sugars, including oligosaccharides, together with fructosan as being immediate energy sources for bacterial growth. These two fractions are sometimes referred to as 'available carbohydrates.' The original 'available carbohydrates' in the grass dry matter amounted to 15·1 per cent, whereas the residual 'available carbohydrates' in the resulting silages ranged from 1·8 to 2·5 per cent.

The most variable constituents in the carbohydrate group were the hemicelluloses, the values for individual components being generally lower than in the original grass.

Two cellulose determinations were carried out, one being a true cellulose consisting entirely of glucosan, determined after hydrolysis by chromatographic separation of glucose as outlined previously. The other cellulose was determined by the Crampton and Maynard (C. & M.) method (20) and is an acid-insoluble organic residue. It is difficult to explain the difference between these cellulose results, especially in silage from silo D, although it is known that Crampton and Maynard cellulose is not pure glucosan. A sample of cellulose prepared from Italian ryegrass by the Crampton and Maynard method was hydrolysed and a chromatographic analysis of this hydrolysate showed that arabinose and xylose were present as well as glucose ; the original cellulose also contained 0·26 per cent N.

Lignin, although not a carbohydrate, is included in the total carbohydrate values because of its association with this group of compounds.

Since a fairly comprehensive analysis of grass and silages has been undertaken in this work, it is interesting to total the individual constituents. The addition of ash, ether extract, total carbohydrates including lignin, acids, protein nitrogen $\times$ 6·25, non-protein non-volatile nitrogen $\times$ 6·25 and volatile nitrogen expressed as ammonia give the following percentage values for grass 97·9 ; and silages A, B, C and D respectively 86·5 ; 87·8 ; 90·0 ; 83·3. In the case of the silage values, a certain degree of error occurs owing to the inclusion of some of the acetic acid in the ether extract fraction. This error will not be great, however, because the determination was carried out on the dried material from which the majority of the volatile acids had been driven off. A further considerable error may be introduced by using the factor 6·25 for conversion of non-protein non-volatile nitrogen to amino acids. This factor is likely to be on the low side.

The fact that about 98 per cent of the constituents in the dry matter of the grass and only about 87 per cent of the silage compounds have been accounted for, suggests that there are considerable amounts of other substances in silage not identified in this work, probably produced by carbohydrate breakdown. Hirst and Ramstad (21) have studied the non-nitrogenous non-volatile organic acids in extracts of ryegrass and silages and have shown that increases in quinic and succinic acids can occur although malic and citric acids tend to disappear during ensilage. It seems that the total quantities of these acids in silages is likely to be lower than in the original herbage however. Ferguson (22) has stated that although herbage samples commonly contain 4-7 per cent organic acids calculated as malic acid on a dry matter basis, some 2-3 per cent are uncombined. Tests also showed that the free acids were only partly soluble in petroleum ether. In our silage studies pectic substances were not determined although Ferguson (22) has stated that there are usually between 1·0 and 1·5 per cent in dried herbage samples. Alcohol has been isolated from silage by many workers. Watson (2) has stated that the average content of alcohol in fresh silage lies in the neighbourhood of 0·3 per cent. It is clear that this volatile compound if present in any quantity would affect the corrected dry matter values. Alcohol was not found in any of the four silages studied in this work. Waite and Gorrod (48, 49) have recently carried out a comprehensive analysis of ryegrass and in addition to the constituents examined in our studies, determined the organic acids, phenolic compounds, pectin complex, uronic anhydrides and acetyl. From these detailed investigations 97-98 per cent of the constituents in the grass dry matter were identified. The composition of the effluents obtained from the four silos over the 63 day ensiling period are given in Appendix I. The dry matter contents of the effluents followed a similar pattern, the mean values ranging from 5·6 per cent on the 3rd day to 9·0 per cent on the 63rd day. The N values over a similar period ranged from ·215 to ·445. The acetic acid and ash contents also increased over this period.

The graph in Figure 2 shows the pH pattern of the effluents. The most interesting feature of this is the low values recorded for the early effluents obtained—these avaraged 4·4 for the four silos and declined, with some fluctuations, to a pH of 4·0. Although the effluent pH can be taken as a guide to the fermen-

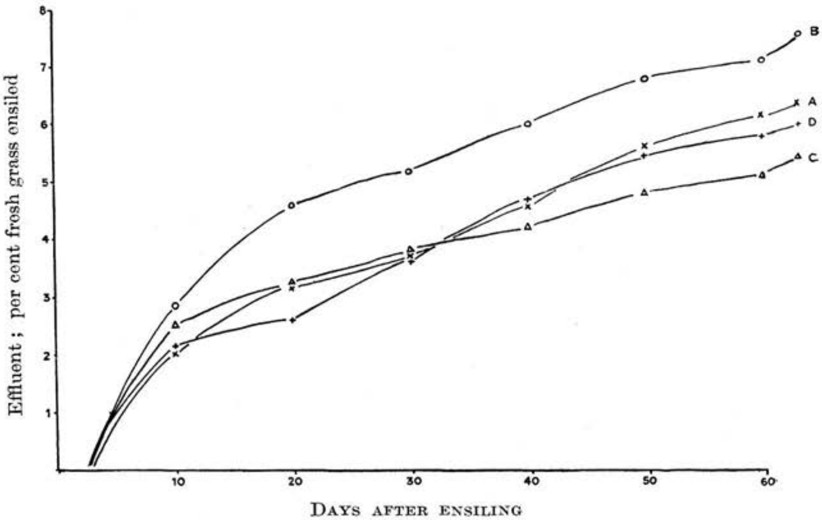

Fig. 3. Weight of effluent as percentage weight fresh grass ensiled. Expt. 1

tation reaction which occurred within the silo, it is difficult to relate the effluent pH value to that of the mass of silage as a whole since the effluent is not a representative extract of the silage but only that of the bottom layers.

LOSSES

Throughout the experiment the silos were weighed daily. The daily effluent losses are shown in Figure 3. The gaseous losses (*i.e.* total loss—effluent loss) at the end of the 63rd day were only 0·61, 0·67, 0·88 and 0·59 per cent for silos A, B, C, and D respectively. The corresponding losses in effluents were 6·46, 7·56, 5·43 and 5·99 per cent.

The detailed losses of individual constituents are given in Table 2. In all four silos the dry matter losses were low and ranged from 6·6 to 8·2 per cent. The mean N loss was 6·3 per cent, including 4·1 per cent recovered in the effluent. The average protein breakdown amounted to 67 per cent and

TABLE 2

EXPERIMENT 1

Percentage losses during ensilage

	A		B		C		D	
	Total	Effluent	Total	Effluent	Total	Effluent	Total	Effluent
Total	7·1	6·5	8·2	7·6	6·3	5·4	6·6	6·0
Dry matter	7·0	2·6	8·1	3·0	6·6	2·2	8·2	2·4
Crude protein	8·0	4·2	6·3	4·8	6·8	3·5	4·1	3·8
Ether extract	+29·5	—	+22·0	—	+29·6	—	+26·2	—
Crude fibre	+ 3·4	—	+ 1·9	—	+ 1·8	—	+ 0·8	—
N.F.E.	18·3	—	19·5	—	17·2	—	20·0	—
Total N	8·0	4·2	6·3	4·8	6·8	3·5	4·2	3·8
Protein N	69·2	—	66·5	—	68·0	—	63·8	—
Total carbohydrates incl. lignin	40·4	—	40·7	—	35·4	—	46·3	—
Sugars	78·5	1·9	84·6	2·6	80·3	1·8	85·5	2·2
Fructosan	95·5	—	95·7	—	96·2	—	95·1	—
Cellulose (C. & M.)	0·7	—	0·5	—	+ 0·5	—	0·4	—
Cellulose (glucosan)	7·7	—	17·3	—	17·0	—	25·3	—
Hemicelluloses	54·4	—	34·8	—	19·5	—	45·1	—
Galactan	75·0	—	31·0	—	6·4	—	52·1	—
Araban	75·7	—	70·2	—	21·2	—	57·1	—
Xylan	47·1	—	26·1	—	28·2	—	41·2	—
Lignin	36·1	—	38·0	—	31·0	—	37·0	—
Water	7·1	7·4	8·3	8·6	6·2	6·2	6·2	6·8

although individual amino acids were not determined it is likely that much of the protein hydrolysis did not go beyond this stage. The total carbohydrate losses, including lignin, averaged 40·7 per cent and of these, the highest losses occurred in the free sugar and fructosan fractions. The mean free sugar loss amounted to 82·2 per cent but, because of the breakdown of polysaccharide material during ensilage and owing to the presence of free pentoses in the silage extracts, it is obvious that a greater breakdown of the original sugars in the grass had actually occurred.

It is interesting that the breakdown of hemicelluloses was very much greater than the amount of residual sugars present in the silages; these polysaccharides may play an important part in fermentation reactions. A number of workers including Langston *et al.* (23), McDonald and Purves (24) have noted that in silage studies the total volatile acids and lactic acid production is greater than the loss in total sugars. It is clear from these studies that unless detailed chromatographic analysis of the sugar fraction is carried out, a simple sugar determination of grass and silage extracts is misleading since the latter contain products from pentosan hydrolysis which were not present in the original grass.

It is difficult to assess at this stage to what extent carbohydrates other than soluble sugars and fructosan play a part in acid production. The apparent high breakdown of the lignin fraction is surprising and confirms the unreliability of using this component as an indicator in silage balance studies. It should be noted, however, that the lignin residue as determined in these studies, is not considered to be a 'true' lignin value (Harwood, 17).

Acetic acid was the only volatile fatty acid present in all four silages and about 5·5 per cent of this acid produced was found in the effluent. Although lactic acid determinations were not carried out on the effluents, it is reasonable to assume that the lactic acid loss in this way was of a similar low order. It can be seen from Table 2 that in silos A, B, and D more water was recovered from the silos than was actually present in the original grass; the net gain in water for these silos respectively was 2·23, 2·85 and 4·74 kg. This water presumably was a product of respiration. Little significance, however, can be attached to the actual values since it is impossible to assess the quantity of water used in the hydrolysis of proteins, polysaccharides and other compounds.

NUTRITIVE VALUE

The nutritive values of the grass and silages, as expressed in terms of digestibility coefficients and digestible nutrients, are given in Table 3. The outstanding feature of these results is the similarity between the grass and silages. It is doubtful if much significance can be attached to the low value for the ether-extractable material from silage A.

TABLE 3

EXPERIMENT 1

Percentage digestibility (D.) and percentage digestible nutrients (D.N.)

	Grass		Silages							
			A		B		C		D	
	D.	D.N.	D.	D.N.	D.	D.N.	D.	D.N.	D.	D.N.
Dry matter .	74·0	—	71·0	—	70·7	—	70·6	—	72·2	—
Organic matter	76·7	68·9	75·1	66·3	74·7	66·1	74·5	65·8	76·2	67·5
Crude protein	77·8	14·6	75·2	13·9	76·0	14·5	76·0	14·2	77·4	15·1
Ether extract .	63·5	2·2	58·0	2·8	69·0	3·2	72·3	3·5	68·8	3·3
Crude fibre .	78·1	18·4	81·3	21·3	79·7	20·8	78·0	20·1	81·2	21·6
N.F.E. . .	77·7	34·2	72·7	28·2	71·5	27·6	71·7	28·0	73·0	28·0
S.E. . . .	—	63·7	—	60·3	—	60·6	—	60·6	—	62·0
T.D.N. . .	—	72·2	—	69·7	—	70·1	—	70·1	—	71·6

The digestibility coefficients of the N.F.E. fraction for the silages are rather lower than the value for the original grass; presumably this is due to the losses during fermentation of the more soluble and digestible carbohydrates. This result is also reflected in the starch equivalent (S.E.) and total digestible nutrients (T.D.N.) values, although the energy values calculated for these silages can still be regarded as high.

The limitation of assessing the nutritive value of silages solely in terms of digestible crude protein (D.C.P.) is reflected in the nitrogen balance table shown in Appendix I, where the utilised digestible nitrogen values for the silages are all lower than those obtained for the grass. However, in view of the many variable factors, including liveweight and dry matter intakes, and owing to the fact that endogenous and exogenous nitrogen measurements were not made in these trials, too much significance cannot be attached to these results. It is interesting, however, to note the low and sometimes negative values which occurred for some animals. It is possible, where ruminant animals exist on a silage diet for any length of time, that the combination of high non-protein nitrogenous compounds and low soluble sugars is a distinct disadvantage to the rumen microflora. This may have some application where stock are 'self-feeding' on silage.

Bacteriological Results

Bacterial counts from the grass and silage macerates of Experiment 1 are given in Table 4.

Most of the silage bacteria, except the strict anaerobes, are able to grow on the glucose yeast agar medium, which thus yields a count approximating to a total count of the bacteria in the silage other than the strict anaerobes. Sometimes, however, acetate agar, the selective medium designed to give counts mainly of lactobacilli, yields counts which are higher than those obtained on glucose yeast agar. This occurs when lactobacilli become dominant in the silage and it is occasioned by the development on the acetate agar of certain types of lactobacilli which do not produce colonies of countable size on the less complex medium.

TABLE 4

Experiment 1

Counts of bacteria from grass and silages

	Bacterial count (millions/g. dry wt. grass) on			of	
	glucose yeast agar	acetate agar	lactate agar	anaerobes	pH
Fresh grass	690	2·5	24	—	6·3
Laboratory-made silage					
3 days at 30°C	3300	3600	<0·01	<0·01	4·18
7 ,, ,, ,,	1600	1500	<0·01	0·09	4·04
63 ,, ,, ,,	22	21	<0·01	<0·01	3·92
95 ,, ,, ,,	6·6	6·6	<0·01	<0·01	3·90
Experimental silage (after 63 days)					
Silo A	700	900	0·26	0·14	3·8
Silo B	1300	1300	6·9	6·6	3·7
Silo C	1100	1300	0·02	0·06	3·9
Silo D	>550 m	900	<0·01 m	0·01	3·8
Waste	12000	5100	m		

m = growth of moulds.

Fresh herbage.—The bacterial flora of the fresh herbage consisted for the greater part of the aerobic organisms generally associated with grass. These comprised Gram-positive coryneform types and Gram-negative bacteria which produce yellow growths on agar, together with a smaller number of *Pseudomonas* spp. and other Gram-negative organisms. The acetate agar yielded a relatively small count of lactobacilli, but nevertheless a count significantly greater than is often found on such herbage (Stirling, 25).

Laboratory-made silage.—In the laboratory-made silage held at 30°C lactobacilli became dominant within the first three days during which time the pH value fell to 4·18. At this stage of the fermentation streptococci were demonstrable in comparatively low number (1·5 million/g. dry weight of grass) on glucose yeast agar incubated at 45°C, a temperature which prevents the growth of the majority of the other bacteria. By this time Gram-negative bacteria had almost disappeared; only a few colonies developed on lactate agar which was inoculated with undiluted silage macerate. At later examinations of the laboratory silages no colonies appeared on this medium thus indicating that the Gram-negative types had died. Lactobacilli remained the dominant organisms up to the last examination after holding for ninety-five days, when the pH was 3·9. Anaerobes were shown to develop between three and seven days but their number remained small throughout the period of the experiment. The pH of the silage was sufficiently low to suggest that there could be little development of the anaerobic bacteria.

Experimental silages.—The four large silos all yielded good silage with a low pH, in each of which lactobacilli were the dominant organisms. The sample from silo B had the lowest pH and the highest count of lactobacilli; it also gave a higher count of anaerobes than the other three silages. The counts on glucose yeast agar and on lactate agar from silo D could only be approximate because the growth of the bacteria on these media was to some extent masked by the development of moulds. The presence of the moulds was in all probability due to the inclusion of a small quantity of the top (waste) material during the sampling of the silage. The bacterial counts of waste material were very high, as is shown in the same table, and moulds were nearly equal in number. The inclusion of spoiled silage could also provide an explanation for the variability of the counts of anaerobes, for example the high count of anaerobes from silo B in which the pH was so low as to suggest that there could have been little development of anaerobes throughout the bulk of the silage. The difficulty experienced in gathering a truly representative small sample for bacteriological examination from a large quantity of silage is well recognised, therefore the close similarity of the bacterial counts and the final pH values of the samples from the four experimental silages seems to indicate that the course of the fermentation in each silo had been the same.

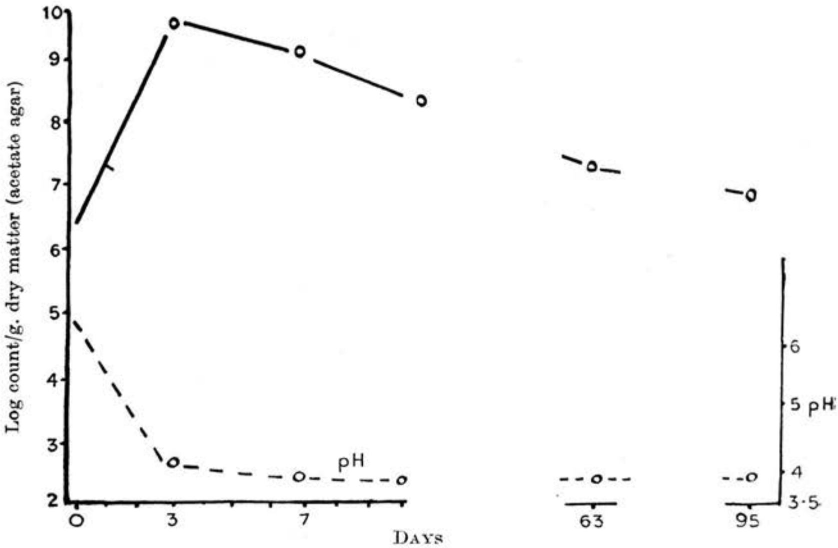

FIG. 4. Lactobacillus counts and pH values obtained from laboratory-made silage in Expt. 1

Lactobacilli in the silage.—The outstanding feature of the fermentation in the laboratory-made silages was the rapid development of lactobacilli and quick fall of pH as shown in Figure 4. A representative selection of lactobacillus colonies from plates inoculated with the fresh herbage gave seven heterofermentative types and three homofermentative types, while at the time of the last examination of the laboratory-made silage the dominant lactobacillus appeared to be the homofermentative *Lactobacillus plantarum*. Isolations from silos A, B, C and D yielded both homofermentative and heterofermentative types of lactobacilli. It seems likely that the development of lactobacilli had been much the same in both the laboratory-made and the larger experimental silages.

EXPERIMENT 2

Experimental

THE purpose of this experiment was to study the effect of consolidation upon ensilage. It has previously been mentioned that consolidation, as interpreted in silage studies, is dependent on the two factors—compaction during filling and compression or weight applied to the grass surface after filling. In this experiment only the latter was varied.

The grass used in this experiment was obtained from the same field as in the previous experiment and consisted of pure Italian ryegrass. The grass was cut with a mowing machine and ensiled in the four silos with tramping, on 3rd October 1957. Similar quantities of herbage were ensiled in each silo up to the 160 cm. level. After ensiling, the grass was covered with polythene sheeting and compression discs in the usual way. The same quantity of grass—viz. 907 kg.—was added in each silo, the consolidation weights applied were 678, 445, 228 and 26 kg. to silos A, B, C and D respectively. These weights corresponded to pressures of 36·9, 24·2, 12·4 and 1·4 g./sq. cm. The consolidation weights applied to silo A (control) were similar to those used in the first experiment. In addition to the usual recordings, temperature measurements were also made. The positions of the thermocouples at the commencement of the experiment were at the following depths :—

1. 35 cm. central.
2. 35 cm. outer.
3. 70 cm. central.
4. 70 cm. outer.
5. 105 cm. central.
6. 105 cm. outer.
7. 140 cm. central.
8. 140 cm. outer.
9. 155 cm. central.

The thermocouples placed in the outer position were situated about 20 cm. from the silo wall.

The silos were opened on the 22nd February 1958, *i.e.* 143 days after filling. The mouldy material considered as unfit for feeding to stock was discarded after weighing. The quantities of this ' spoiled ' material were as follows :—

	Weight of ' spoiled ' material kg.	*% of original grass ensiled*
A	Nil	Nil
B	118·95	13·11
C	113·05	12·46
D	174·34	19·22

Results and Discussion

The relative density of the grass in this experiment was the same as in Experiment 1 (0·77) so that the degree of compaction during filling in all four silos was the same as in the previous experiment. The consolidation weights, including discs, applied in A amounted to 678 kg. This corresponds to a pressure of 36·9 g./sq. cm. and could be regarded as similar to a pressure obtained from an 8 in. (20 cm.) layer of moist soil. It is customary under practical conditions to use soil for consolidation purposes and generally a layer of 6-8 in. is recommended. No stone blocks were applied to the surface of silo D and the 26 kg. weight mentioned previously was therefore solely due to the polythene sheeting and wooden discs.

Figure 5 shows the change in volume occupied by the silage during the experiment. It is obvious from this graph that the rate of fall of the levels varied in different silos and was directly proportional to the consolidation weights applied to the surface. The graph for silo A was similar to that obtained for the same silo in the previous experiment.

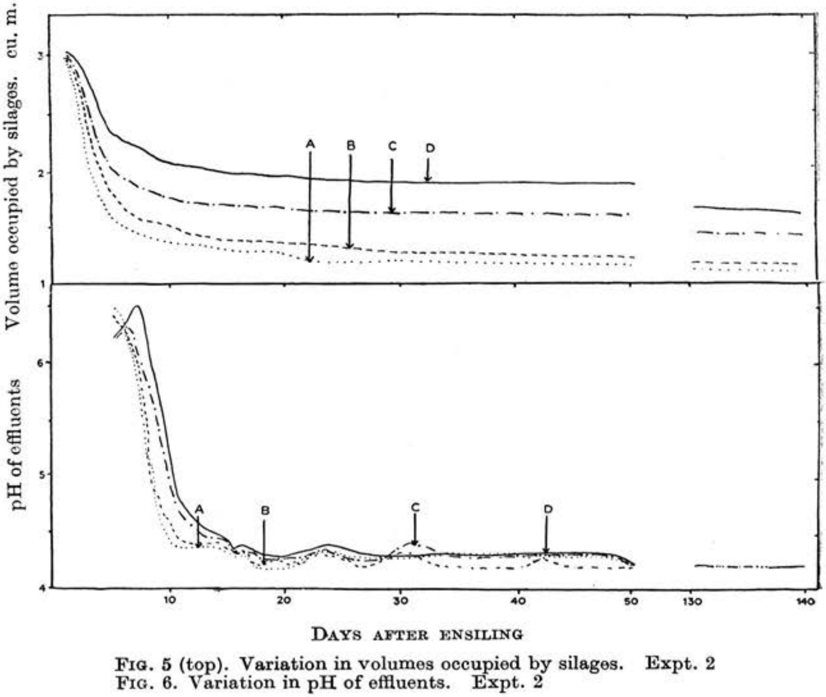

Fig. 5 (top). Variation in volumes occupied by silages. Expt. 2
Fig. 6. Variation in pH of effluents. Expt. 2

COMPOSITION

Table 5 shows the chemical composition of the grass and silages. The silage results shown in this table are for samples representative of the whole material excluding waste. The average pH values of the four silages were dissimilar and ranged from 4·3 in A to 4·9 in D.

In this experiment the silo contents were sampled in

TABLE 5

EXPERIMENT 2

Composition of grass and silages (per cent dry matter)

	Grass	Silages A	Silages B	Silages C	Silages D
*Dry matter	19·92	19·08	17·86	17·79	17·70
incl. volatile acetic acid		0·26	0·36	0·28	0·28
,, ,, lactic acid		0·25	0·25	0·22	0·27
,, ,, ammonia		0·12	0·07	0·04	0·18
Organic matter	89·2	88·2	87·6	87·7	87·7
Crude protein	19·0	21·9	23·4	22·7	22·8
Ether extract	4·5	5·6	5·2	5·0	5·3
Crude fibre	20·8	23·7	24·0	23·7	23·9
N.F.E.	44·9	37·1	35·0	36·3	35·8
Total N	3·04	3·51	3·74	3·64	3·64
Protein N	2·73	1·28	1·37	1·18	1·15
Non-protein N	0·31	2·23	2·37	2·46	2·49
Volatile N		0·45	0·45	0·50	0·62
Total Carbohydrates incl. lignin	51·5	38·6	34·6	38·8	35·6
Total Sugars	9·1	2·9	1·3	2·8	2·0
Sucrose	4·5				
Glucose	1·6	Present in all silages in small amounts.			
Fructose	1·3				
†Oligosaccharides	1·9				
Galactose					
Arabinose					
Xylose					
Fructosan	7·0	0·04	0·05	0·05	0·05
Total Hemicelluloses	10·0	10·7	10·2	8·8	8·4
Galactan	0·86	0·58	0·58	0·70	0·48
Araban	2·85	1·84	1·44	0·95	2·21
Xylan	6·3	8·3	8·2	7·2	5·7
Cellulose (C. & M.)	23·2	25·5	26·8	25·5	25·8
Cellulose (glucosan)	20·2	19·3	18·2	22·4	20·2
Lignin	5·2	5·7	4·8	4·8	5·0
Lactic acid		10·4	10·7	10·2	9·1
Acetic acid		1·56	2·22	1·66	1·80
*pH		4·3	4·5	4·35	4·9

* pH and volatile constituents determined on fresh material.
† Excluding sucrose.

six fractions, the first sample being representative of the top layer which in the case of B, C and D represented the mouldy or ' spoiled ' material. Dry matter and pH determinations only were carried out on these samples, the former were not corrected for volatile losses. The results are given in Table 6.

TABLE 6

EXPERIMENT 2

Dry matter (uncorrected) and pH values of samples of silage

Sample	Silages							
	A		B		C		D	
	pH	% D.M.	pH	% D.M.	pH	% D.M.	pH	% D.M.
1	4·7	17·89	*7·1	14·81	*6·3	15·53	*7·6	13·99
2	4·3	19·45	5·2	17·07	5·3	17·50	6·4	16·69
3	4·2	18·82	4·7	17·67	5·0	18·14	5·5	17·35
4	4·2	18·47	4·2	17·07	4·2	17·27	5·3	18·04
5	4·2	18·05	4·2	18·24	4·2	17·76	4·3	16·92
6	4·2	17·73	4·2	18·16	4·2	17·67	4·2	17·34

* Representative of ' spoiled ' silage.

Considerable variation in pH occurred throughout the silage mass and although the silage samples from the lower half of the silos were well preserved, the amount of badly preserved material at the top of the silos was related inversely to the consolidation weights applied. From Table 6 it appears that the wettest material occurred in the top layers of the silage. Too much significance, however, cannot be attached to this because the volatile losses on drying were likely to be high in these samples of high pH value. The dry matter values given in Table 5 were representative of the whole material excluding ' spoiled ' silage, and had been corrected for volatile losses.

The total N values of the silages were much higher than the N contents of the original grass. This was largely a reflection of the high losses of soluble carbohydrate which occurred. The crude fibre results showed similar trends. The volatile N content of silage D was about 37 per cent higher than that of silage A. This could be expected in view of the higher pH value of this silage. The results also indicated that a greater breakdown of protein had occurred in silos C and D than in A and B.

Langston *et al.* (23) have stated that the crude protein content (percentage) of a silage was not a reliable criterion of overall quality nor of ensiling efficiency since they found in their studies that silages showing the highest content and greatest gain in percentage of crude protein were otherwise of low quality and poorly preserved. Our results confirm this finding and in view of the almost complete recovery of total N in these experiments the ratio of total N in original grass to total N in resultant silage does give an indication of dry matter loss, provided the effluent N is not excessive.

Although chromatographic separation of sugars in the silage alcohol extracts was carried out, individual sugars were not quantitatively determined. Glucose, xylose, fructose, arabinose, galactose and traces of oligosaccharides were present. The 'available' carbohydrate present in the original grass was 16·1 per cent, *i.e.* slightly higher than that present in the July grass. The fructosan contents of the silages were again very low.

There were considerable variations in the amounts of individual hemicelluloses present in the silages. The xylan content decreased with decreasing consolidation weights and araban followed a similar trend in silos A, B and C. It is difficult to explain the high value for this pentosan in silo D however. There was again a difference in the two cellulose figures; here the Crampton and Maynard cellulose was higher than the pure glucosan cellulose.

As in the previous experiment, individual components of the dry matter were totalled and gave the following percentage values for grass 85·8; and silages A, B, C and D respectively 87·6, 86·2, 88·2 and 83·8. Whereas in the first experiment 98 per cent of the dry matter constituents were accounted for, in the autumn cut grass about 14 per cent of the organic components were not identified.

The composition of the effluent samples is given in Appendix I.

In this experiment effluents were collected daily and aliquot samples were bulked over weekly periods. These were stored under toluene in a refrigerator during the weekly collection periods, at the end of which, samples were taken for analysis. As in the previous experiment the dry matter, nitrogen, ash, and acetic acid contents increased over the 143 days ensiling period, while the sugar values decreased. A number of sugar chromatograms were run on effluent samples. Exact quantitative measurements were not made ; the values reported below were determined from the density of the chromatographic spots as estimated visually. Effluents from the four silos showed similar results and the general trend is shown in Table 7. Treatment prior to chromatographic separation was as follows :—

A 10 ml. portion of the effluent was deionised by shaking for one hour over a mixed resin bed of Amberlite IR 100 : IR 4B. Chromatograms were run in ethyl acetate : acetic acid : water (3 : 1 : 3) and benzene : butanol : pyridine : water (1 : 5 : 3 : 3). Table 7 shows the results up to the 13th week when this aspect of the experiment was discontinued.

TABLE 7

EXPERIMENT 2

Sugar analysis of the effluents

Weeks	Gal.	Glu.	Arab.	Fruc.	Xyl.	Rib.
1	+	+++	Tr.	++	Tr.	Tr.
3	++	+	Tr.	++	Tr.	—
5	+	+	Tr.	+	+	—
7	+	+	+	+	+	—
9	+	—	+	+	+	—
11	++	—	+	++	++	—
13	++	—	+	++	++	—

It can be seen from Table 7 that glucose had disappeared from the effluent by the seventh week, while arabinose and xylose had not appeared in any quantity until the fifth-seventh week. These results suggest that the breakdown of hemicelluloses in any great quantity had not occurred until some weeks after ensiling.

The graph in Figure 6 shows the pH pattern of the effluents and although the general trends were similar, the rate at which the pH values fell over the first fifteen days was related to the consolidation weights applied. One important feature of this experiment compared with the previous one is that it took much longer for the pH values to fall. Previously the samples of effluent collected on the third day had a pH value of 4·4. In the second experiment it took about eleven days before the effluent pH reached this value in silo A. The significance of this is discussed later.

LOSSES

The daily gaseous and effluent losses are shown in the graphs in Figures 7 and 8. The percentage gaseous losses at the end of the 143 day ensiling period amounted to 1·44 ; 1·78 ; 1·74 ; and 1·97 for silos A, B, C and D respectively.

On the 63rd day the corresponding gaseous losses were 1·26 ; 1·46 ; 1·45 and 1·69. The gaseous loss from silo A in the previous experiment over a 63 day period was only 0·61 per cent. The corresponding percentage effluent losses in the second experiment over 63 days were 9·64 ; 7·58 ; 4·50 and 4·28. In the previous experiment the effluent loss from silo A was 6·46 per cent of the original weight of herbage ensiled.

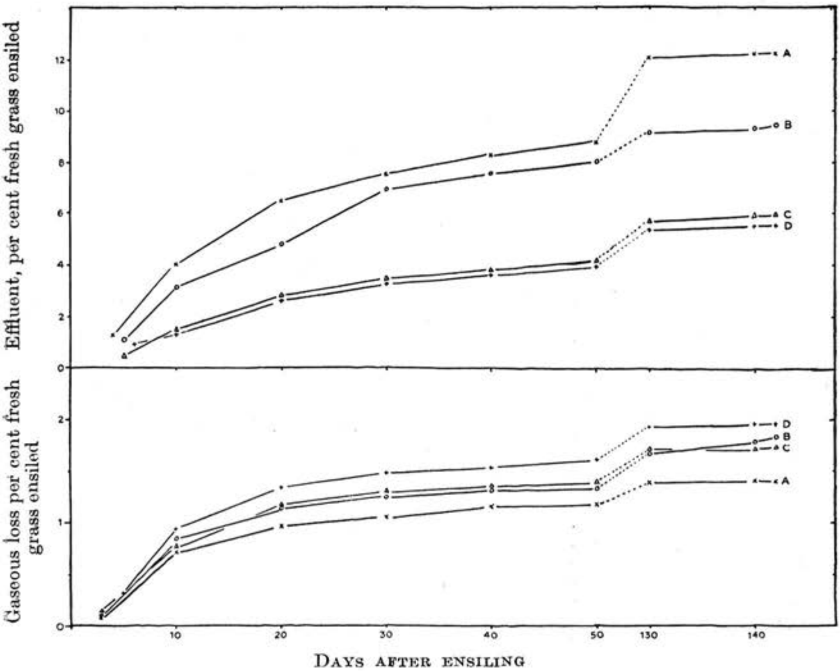

FIG. 7 (top). Weight of effluent as percentage weight fresh grass ensiled. Expt. 2
FIG. 8. Gaseous loss as percentage fresh grass ensiled. Expt. 2

The detailed losses of individual constituents are given in Table 8. The dry matter losses ranged from 17·4 per cent in silo A to 20·5 per cent in silo B. In silo A, 4·3 per cent dry matter was lost via the effluent. It is obvious from these results and also from the high pH values of early effluent samples, that a different type of fermentation had occurred in the autumn grass from that which had occurred in the summer herbage.

As in the previous experiment the losses of nitrogenous compounds during ensilage, within the limits of experimental error, were recovered in the effluents. The protein breakdown ranged from 60 per cent in silo B to 65·3 per cent in silo D.

Considerable variation in total carbohydrate breakdown occurred in different silos, the value for silo A being slightly lower than in the previous experiment. Here again the main breakdown occurred in the fructosan and sugar fractions. The apparent xylan gain in silos A and B is difficult to explain, and must be due to errors in separation of xylose and arabinose during chromatographic analysis. Lignin was again apparently broken down with the highest loss occurring in silo B.

TABLE 8

EXPERIMENT 2

Percentage losses during ensilage

	A		B		C		D	
	Total	Effluent	Total	Effluent	Total	Effluent	Total	Effluent
Total	13·8	12·3	11·3	9·5	7·7	6·0	7·5	5·5
Dry matter	17·4	4·3	20·5	3·4	17·6	2·1	17·8	1·9
Crude protein	4·7	7·3	2·1	5·8	1·3	3·8	1·4	3·3
Ether extract	+2·6	—	8·0	—	7·3	—	3·3	—
Crude fibre	6·0	—	8·0	—	5·9	—	5·5	—
N.F.E.	31·8	—	34·3	—	33·5	—	34·6	—
Total N	4·7	7·3	2·0	5·8	1·3	3·8	1·3	3·3
Protein N	61·3	—	60·0	—	64·5	—	65·3	—
Total Carbohydrates incl. lignin	38·1	—	46·7	—	37·9	—	43·2	—
Sugars	74·0	6·1	88·6	5·7	74·6	3·2	81·9	2·9
Fructosan	99·5	—	99·5	—	99·5	—	99·5	—
Cellulose (C. & M.)	9·1	—	8·2	—	9·3	—	8·6	—
Cellulose (glucosan)	21·2	—	28·5	—	8·6	—	17·7	—
Hemicelluloses	11·4	—	19·0	—	16·2	—	31·5	—
Galactan	43·9	—	46·5	—	26·9	—	54·2	—
Araban	46·6	—	59·8	—	72·4	—	36·3	—
Xylan	+9·1	—	+3·3	—	6·1	—	26·2	—
Lignin	9·5	—	26·5	—	24·7	—	21·1	—
Water	12·9	14·3	9·0	11·0	5·3	6·9	4·9	6·4

In all four silos more water was recovered than was present in the original grass. The net gain in kg. was 10·7 ; 14·7 ; 12·1 and 10·9 for silos A, B, C and D respectively. All these values were very much higher than in the previous experiment and may reflect the high gaseous losses which occurred.

Temperature changes

In this experiment temperature measurements were recorded daily from the nine thermocouples in each silo. Air temperatures were also recorded by means of an automatic thermograph situated near the silos. In silos A, B, C and D the maximum mean temperatures recorded were 18° ; 18° ; 17° and 18°C respectively, all on the eighth day. The maximum individual temperatures recorded were 21° for silo A on the second day, a similar value for silo B on the third day, 19° for silo C on the second day and 26° for silo D on the fifth day. In every case the higher temperatures were recorded in the top layers of herbage, *i.e.* by those thermocouples originally positioned between the 140-160 cm. levels.

The mean temperature values, after the initial rise, showed a similar pattern to that of the mean air temperature. The temperature in silo D was always about two degrees higher than in the other silos and this was maintained even after 140 days. This slightly higher temperature in silo D could have been due to heat being produced in the top layers of material caused by continued microbiological activity.

Nutritive value

The nutritive value of the autumn cut grass and the silages produced from this grass is given in Table 9. As in the previous experiment, the digestibility values of the silages did not differ greatly ; the digestion coefficients of the crude protein were slightly higher for the silages than for the original grass. The reverse finding was true for the N.F.E. fraction. It appears from these results that the nutritive value of the silages was similar to that of the original herbage, with a very small reduction in net energy value.

Nitrogen balance studies were carried out as in the previous experiment (Appendix I). The mean retention value, expressed as per cent digestible N retained, from four sheep on the grass diet was 19·8 and the values for three sheep on each of the silage diets, A, B, C and D, were 10·2, 18·3, 17·5 and 19·1 respectively. As in the previous experiment, these values are low and may reflect the high protein content of the diet. The intake of C.P. per sheep on the silage diets approximated to about 0·5 lb. which is equivalent to about 0·38 lb. D.C.P. per day. According to Watson and More (26) about 0·2 lb. digestible protein per head per day is sufficient for maintenance for fattening lambs of ordinary size. It is clear from this, that nitrogen balance studies on high protein foods are likely to give low estimates of nitrogen utilisation and the absolute figures can only be used for comparison with rations of similar C.P. intake.

TABLE 9

Experiment 2

Percentage digestibility (D.) and percentage of digestible nutrients (D.N.)

	Grass		Silages							
			A		B		C		D	
	D.	D.N.	D.	D.N.	D.	D.N.	D.	D.N.	D.	D.N.
Dry matter .	73·6	—	71·3	—	72·5	—	74·0	—	72·3	—
Organic matter	76·8	68·5	75·8	66·9	76·0	66·6	77·2	67·7	75·9	66·5
Crude protein	70·9	13·3	76·9	14·2	79·0	15·1	79·6	14·9	78·3	15·3
Ether extract .	59·9	2·1	63·5	3·1	65·8	3·0	69·3	3·4	65·1	3·1
Crude fibre .	81·6	19·2	80·7	21·2	81·7	21·4	81·7	21·0	81·4	21·1
N.F.E. . .	78·8	34·7	74·1	28·7	71·7	27·7	74·1	29·0	72·4	27·8
S.E. . . .	—	63·5	—	61·5	—	61·4	—	62·9	—	61·6
T.D.N. . .	—	71·9	—	71·0	—	71·0	—	72·4	—	71·1

Comparison with Experiment 1

In order to compare the losses in the second experiment directly with those in Experiment 1, a number of silage samples were taken from the silos on December 4th, some 63 days after ensiling. This period of time was similar to the total ensiling period of the first experiment.

From silo A, six samples were taken, using a 2 in. corer, three from port No. 1 (bottom port), and three from port No 2. Great difficulty was encountered in sampling from the bottom port as the corer tended to slide between the horizontal layers of silage instead of cutting into the material. In sampling from No. 2 port immediately above it, the corer was forced through the silage at an angle of about 45 degrees. It was found that this was the only satisfactory way of obtaining a sample. In the case of silos B, C and D single core samples only were taken.

It has been mentioned previously that this method of sampling silage *in situ* is unreliable, and during the procedure it was obvious that some residual moisture had been squeezed from the core samples. Furthermore, because of the method necessarily employed with the type of corer available, the samples could hardly be regarded as being representative. The analytical results are, however, shown in Table 10.

TABLE 10

EXPERIMENT 2

Analysis of cored samples removed from silos on 63rd day (per cent dry matter)

	A	B	C	D
*Dry matter (corrected) .	19·24	17·23	17·58	17·79
Total N . . .	3·33	3·73	3·65	3·81
Lactic acid . . .	6·60	5·08	7·45	5·25
Acetic acid . . .	1·11	2·15	1·02	1·56
Butyric acid . . .	0·07	0·57	0·07	0·24
*pH	4·4	5·5	4·9	5·5

* pH and dry matter determined on fresh material.

The losses in dry matter and nitrogen calculated from these results are shown in Table 11.

TABLE 11

EXPERIMENT 2

Percentage losses during ensilage (after 63 days)

	Dry matter		Nitrogen	
Silo	Total	Effluent	Total	Effluent
A	13·9	3·1	5·8	5·3
B	21·3	2·5	3·3	4·2
C	17·0	1·5	0·4	2·7
D	16·0	1·4	+5·5	2·4

Perhaps the outstanding feature of these results was the almost complete recoveries of nitrogen in silos A, B, and C. The results for silo D, however, showed an apparent gain in nitrogen.

Since most samples were taken from silo A and this was the silo which had been ensiled in a similar manner to that in Experiment 1, more importance could be attached to these results. The dry matter of the silage was 19·24 per cent which was slightly higher than the value obtained after 143 days. The lactic acid content of the cored sample was much lower than that of the silage sampled at the end of the experiment, but in view of the possible loss of this acid during coring, too much significance cannot be attached to this finding.

The gaseous losses calculated from the cored and final samples were 1·26 per cent and 1·44 per cent respectively. It can be seen from the graphs in Figures 7 and 8 that although most of the gaseous and effluent losses had occurred at the beginning of the ensiling period, changes continued to occur in the silo up to 142 days after filling.

Bacteriological results

In this experiment a set of laboratory silages was again made. The bacterial counts and pH values of the grass and silage macerates in this second series are given in Table 12.

Fresh herbage.—The bacterial flora of the fresh herbage differed little from that of the previous experiment except for the lactobacillus content which on this occasion was negligible. Only a few colonies developed on acetate agar plates inoculated with undiluted grass macerate.

Laboratory-made silage.—In the laboratory-made silage the count on acetate agar was still low at the examination after five days and it was small in comparison with the count on glucose yeast agar, while the count of Gram-negative organisms remained high. Anaerobes had developed and they were also detected at all subsequent examinations of the silage. Thus the bacterial development was rather different from that of the previous experiment when the lactobacilli became dominant within three days.

The pH of the silage dropped only slowly and was at no time below pH 5. It would seem apparent that the number of lactobacilli (Figure 9) present on the fresh herbage was so small, that in each laboratory silo there were too few to increase sufficiently rapidly to become dominant in the early stages of the fermentation and to bring about a rapid acidification of the silage. The development of other types of bacteria such as the Gram-negative organisms means that there would have been competition for the available nutrients ; more of the carbohydrate would have been transformed to acids other than lactic acid, and to carbon dioxide and hydrogen, while utilisation of lactic acid and formation of basic compounds would have tended to keep the pH at a higher level.

TABLE 12

EXPERIMENT 2

The pH values and bacterial counts from grass and silage

	Bacterial count (millions/g. dry wt. grass) on			of	
	Glucose yeast agar	Acetate agar	Lactate agar	Anaerobes	pH
Fresh grass	520	<0·01	23	—	6·10
Laboratory-made silage					
5 days at 30°C . .	4440	300	2900	5·4	5·88
12 ,, ,, ,, . .	440	260	—	0·9	5·60
60 ,, ,, ,, . .	60	55	<0·01	8·5	5·48
145 ,, ,, ,, . .	42	29	<0·01	5·4	5·10
Experimental silage (143 days)					
Silo A top half . . .	1000	960	0·29	0·02	4·48
bottom . . .	520	720	<0·01	<0·01	4·36
Silo B top half . . .	1300	1600	2	0·01	4·71
bottom . . .	910	1100	<0·01	0·01	4·39
Silo C top half . . .	490	350	1	<0·01	4·98
bottom . . .	490	700	<0·01	<0·01	4·23
Silo D top half . . .	1900	700	49	0·05	5·63
bottom . . .	350	370	<0·01	<0·01	4·52
Silo C waste . . .	>28000	2300	2300	0·5	7·72
Silo D waste . . .	>28000	1000	290	85	8·72
Core samples (63 days)					
Silo A	>800	1900	1·7	<0·01	—
Silo B	5800	2300	290	1·4	—
Silo C	1700	1300	3	0·4	—
Silo D	1700	1900	11	0·5	—

Experimental silage.—When the experimental silos were opened after 143 days, samples were taken separately from the upper and lower halves of the silage. In each silo the upper half showed a higher count of Gram-negative bacteria, and in all but silo C a higher count of lactobacilli. The pH

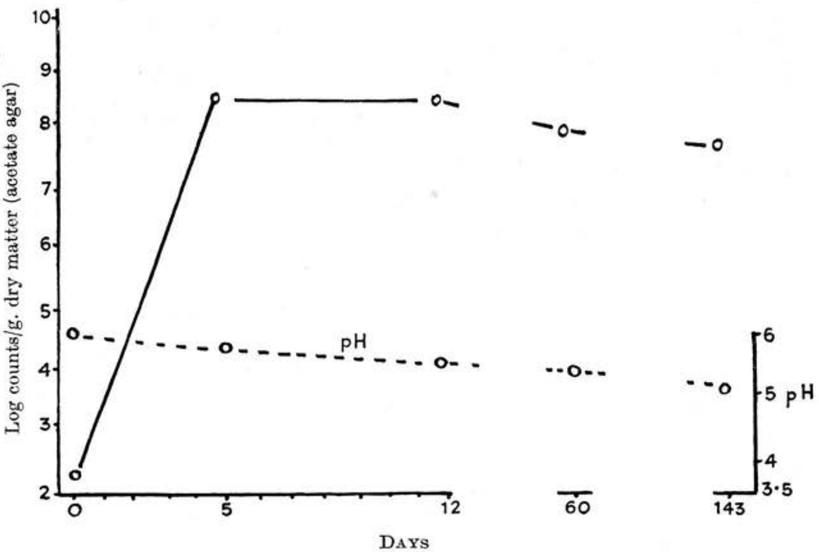

FIG. 9. Lactobacillus count and pH values obtained from laboratory-made silage in Expt. 2

of the lower half was less than that of the upper half. Very high counts of bacteria and moulds were obtained from samples of the waste material which was found at the top of the silos. The counts of lactobacilli were in the same range as that obtained in the previous experiment, but the results from the laboratory silage suggested that these organisms had not been dominant in the early stages of the fermentation. This is borne out by the higher pH figures obtained.

Core samples.—The results of bacteriological examination of core samples from silos A, B, C and D are also shown in Table 12. These samples, taken 63 days after ensiling, all showed higher bacterial counts than those obtained when the silos were finally opened. It is somewhat difficult to assess the value of a core sample for bacteriological examination as much of the juice seems to be squeezed out during the taking of the sample.

Bacteria in the silage.—The bacterial counts of the silage from the experimental silos gave little indication that the fermentation had differed from that of the previous experiment. Homofermentative as well as heterofermentative lactobacilli were isolated both from the large and from the tube-silages. The pH values of the samples from the individual silos appeared to give a better indication of the state of the silage than bacterial counts made on the silage at the time of emptying the silos. The lowest pH was given by the sample from silo A, *i.e.* the silage which had been subjected to the greatest amount of compression. The study of the silages made in the laboratory gave an indication of the probable course of the fermentation in the well-compressed silage of silo A.

EXPERIMENT 3

In a previous experiment (Experiment 2) reference was made to the two important factors associated with consolidation during ensilage; compaction during filling and weight applied to the grass surface after filling, *i.e.* compression. In Experiment 2 the latter was varied and the results indicated that the main effect was shown in the amount of top spoilage produced.

In this experiment the other aspect of consolidation was considered and this was combined with an attempt to produce high temperature silage. In a preliminary trial it had been shown that when the metal silos (153 cm. $\times$ 183 cm.) were filled with unconsolidated grass (*i.e.* without tramping) to the 160 cm. mark, about 650 kg. of herbage were required. From density determinations it can be calculated that the true volume occupied by this herbage amounted to 0·84 cu. m. The volume of the silo up to 160 cm. mark is 3·02 cu. m., therefore the air present amounted to 2·18 cu. m. In the first experiment the calculated air ensiled with the herbage (excluding cellular gases) amounted to 1·84 cu. m.

It is clear from these approximate calculations that the additional air ensiled with the unconsolidated herbage is not very great and it would be unlikely that overheated silage could be produced unless there was an interchange of gases with the atmosphere.

Since one of the objects of this work was to attempt to produce high temperature silage, the injection of oxygen into two of the silos (B and D) during the early stages of the experiment was considered as part of the treatment.

Experimental

The grass used in this experiment was obtained from a pure sward of Italian ryegrass (Lolium italicum) obtained from Boghall Farm, Midlothian. The grass was cut with a mowing machine on 30th July 1958 and ensiled the same day.

The silos were filled up to the 160 cm. level. Silo A was filled in a similar manner to that used in the previous two experiments, *i.e.* employing tramping during filling. The weights placed on top of the herbage were similar to those used in Experiments 1 and 2.

There was no tramping in filling silos B, C, and D. The quantities of grass ensiled and the treatments are shown in Table 13.

TABLE 13

EXPERIMENT 3

Treatments

Date	Silo	Wt. grass ensiled kg.	Volume occupied cu. m.	Consolidation weight kg.	Equiv. Pressure g./sq. cm.
30th July 1958	A	908·6	3·02	679·9	37·4
	†B	664·9	3·02	40·4	2·2
	C	664·1	3·02	27·3	1·5
	†D	664·6	3·02	*38·7	2·1

* On the 16th day the consolidation weight on D was altered to 718·3 kg.

† These silos were insulated with 'Fibreglass' and were injected with oxygen on the following dates :—

Day	Date	Amount of Oxygen injected per silo cu. m.
6	4th Aug.	0·20
10	8th Aug.	0·40
12	10th Aug.	0·20
13	11th Aug.	0·79
	Total	1·59

In an attempt to prevent excessive heat loss through the metal walls of the silos, the outsides of silos B and D were wrapped with a 1 in. layer of 'Fibreglass.' The original consolidation weights in silos B and D shown in Table 13 were obtained from the plastic covering, compression disc and 'Fibreglass.' Silos A and C were not insulated.

During the filling process, thermocouples were placed at different levels in the herbage. Each silo contained nine thermocouples at levels similar to those in Experiment 2 as follows :—

1. 35 cm. central.
2. 35 cm. outer.
3. 70 cm. central.
4. 70 cm. outer.
5. 105 cm. central.
6. 105 cm. outer.
7. 140 cm. central.
8. 140 cm. outer.
9. 155 cm. central.

The thermocouples placed in the outer position were situated about 20 cm. from the silo wall. Temperatures were recorded twice daily until maximum temperature had been reached and then once daily.

On the 6th, 10th, 12th and 13th day after ensiling, oxygen was injected into the herbage mass in silos B and D by coupling an oxygen cylinder to the central effluent drain tube. The volume of oxygen passed into each silo was measured by means of an accurate meter. The volumes of oxygen used are shown in Table 13.

On the 16th day the consolidation weight on top of D was altered to 718·3 kg. The silos were opened on the 23rd September 1958 some 55 days after ensiling. The mouldy material considered unfit for feeding to stock was discarded after weighing. The quantities of the ‘ spoiled ’ material were as follows:—

Silo	*Weight of ‘ spoiled ’ material* kg.	*% of original grass ensiled*
A	23·8	2·62
B	100·9	15·18
C	181·8	27·38
D	78·3	11·78

Results and Discussion

For sampling purposes the silage was divided into seven fractions. Fraction 1 contained the waste or ‘ spoiled ’ material. Only pH determinations were carried out on these samples and the results are shown in Table 14. As was expected the highest pH values occurred in the topmost layers of silage.

The material obtained from the lower part of the silos was in a uniform state of preservation. The mean pH values (excluding spoilage) ranged from 3·7 in silo A to 4·1 in silo C.

TABLE 14

EXPERIMENT 3

pH values of samples of silage

Sample	A	B	C	D
1 (spoilage)	5·2	6·4	6·1	5·8
2	4·3	4·3	4·7	4·2
3	3·8	4·0	4·3	4·1
4	3·7	4·0	4·0	4·0
5	3·7	3·8	3·9	3·9
6	3·7	3·8	3·8	3·8
7	3·7	3·8	3·9	3·8
Bulked	3·7	3·9	4·1	3·8

Figure 10 shows the changes in volume occupied by the silage during the experiment. The consolidation weight applied to silo A was similar to the weight used in Experiments 1 and 2 and the pattern of the graph is similar to the graphs obtained in these previous experiments. Silos B and D followed similar patterns until additional weights were applied to D when the volume decreased rapidly over a period of two days and then fell steadily for the remainder of the ensiling period. The level in silo C did not fall as rapidly as in silos B and D, and this must be attributed to the slightly lower consolidation weight applied to C. The difference between C and B, *i.e.* 13·1 kg., was due to the additional weight on B of the 'Fibreglass' used for insulation purposes. The consolidation weight on C was due entirely to the weight of polythene plus the weight of the compression disc. It is surprising that 13·1 kg. should have made such a difference to the rate of fall of the silage level in silo C but in the initial stages of the experiment this appeared to be the only explanation.

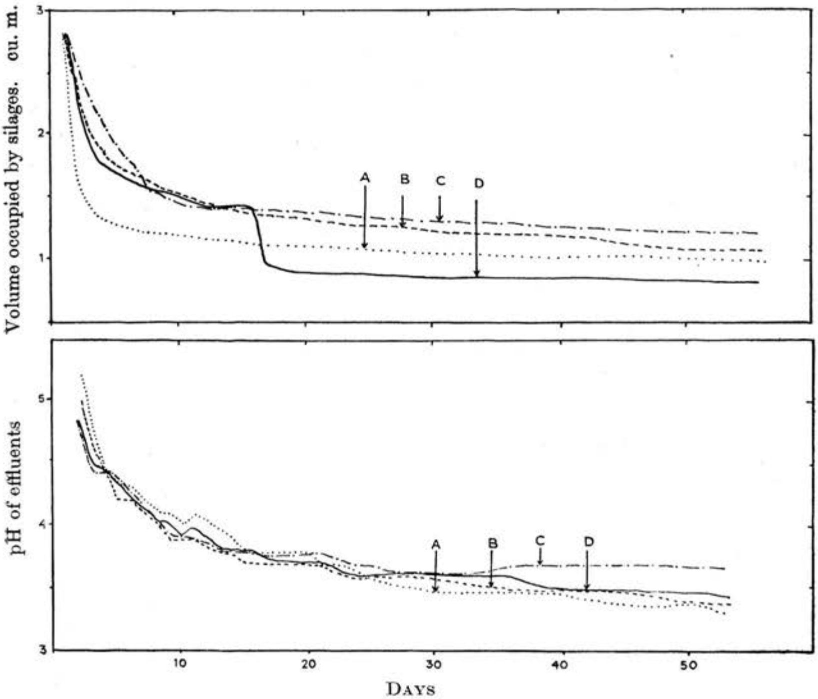

Fig. 10 (top). Variations in volumes occupied by silages. Expt. 3
Fig. 11. Variations in pH of effluents. Expt. 3

COMPOSITION

The chemical composition of the grass and silages is shown in Table 15. The original grass was not of high quality judging from the protein and fibre values. The total sugar and fructosan content amounted to 17·0 per cent, this being slightly higher than in Experiment 1 (15·1) and Experiment 2 (16·1).

TABLE 15

EXPERIMENT 3

Composition of grass and silages (per cent dry matter)

	Grass	Silages			
		A	B	C	D
*Dry matter	14·9	17·42	15·39	14·84	17·06
incl. volatile acetic acid		0·34	0·30	0·28	0·35
,, ,, butyric acid		0·03	0·21	0·33	0·08
,, ,, lactic acid		0·22	0·10	0·05	0·09
,, ,, ammonia		0·03	0·02	0·01	0·05
Organic matter	88·9	86·6	85·5	87·1	86·3
Crude protein	12·0	12·1	13·2	12·5	12·7
Ether extract	2·4	3·5	3·2	3·7	3·3
Crude fibre	26·2	28·9	28·7	29·9	28·4
N.F.E.	48·3	42·0	40·4	40·9	42·0
Total N	1·92	1·93	2·12	2·00	2·03
Protein N	1·71	0·88	1·28	0·94	1·25
Non-protein N	0·21	1·05	0·84	1·07	0·78
Volatile N	0·01	0·14	0·14	0·25	0·19
Total Carbohydrates incl. lignin	61·7	43·9	43·6	44·6	47·7
Total Sugars	7·5	1·16	0·57	0·96	0·69
Sucrose	3·2				
Glucose	1·7	Present in all silages.			
Fructose	1·0				
†Oligosaccharides	1·6				
Galactose					
Arabinose					
Xylose					
Fructosan	9·5	0·37	0·40	0·27	0·42
Total Hemicelluloses	12·6	9·0	11·8	9·5	13·7
Galactan	0·68	0·45	0·38	0·72	0·66
Araban	3·0	1·55	1·33	1·48	2·38
Xylan	8·9	7·0	10·1	7·3	10·7
Cellulose (C. & M.)	27·5	31·0	30·1	31·6	30·4
Cellulose (glucosan)	24·7	26·6	23·3	26·3	25·5
Lignin	7·4	6·8	7·5	7·5	7·4
Lactic acid		11·5	9·2	5·6	8·5
Acetic acid		2·1	2·4	2·1	2·2
Butyric acid		0·18	1·38	2·3	0·47
*pH.		3·7	3·9	4·1	3·8

* pH and volatile constituents determined on fresh material.
† Excluding sucrose.

The low dry matter value of the ensiled herbage reflected the very wet summer of 1958, and particularly the wet period immediately preceding the cutting operation. The large volumes of effluent also reflect this. The dry matter values of the silages were corrected for volatile losses. These losses included acetic, butyric and lactic acids as well as ammonia and, expressed as a percentage of the dry matter values, amount to 3·4 ; 4·0 ; 4·6 ; and 3·3 per cent for silages A, B, C and D respectively.

The residual sugar contents in the silages were again very low. In addition to the hexoses present in the original grass, galactose, arabinose and xylose were again detected. Silage A showed the lowest hemicellulose content, this being mainly due to the lower xylan value. Although the Crampton and Maynard cellulose values were similar in the four silages, the glucosan cellulose in silage B was markedly lower than the values for the other three silages.

Silage A contained the highest lactic acid content with only a trace of butyric acid, whereas the lactic acid in silage C was comparatively low with an appreciable amount of butyric acid also present.

The graph in Figure 11 shows the pH pattern of the effluents. As in the previous July experiment the fall in pH was rapid and by the 4th day the value in all four effluents was 4·4. The minimum value in C occurred on the 30th day and then increased slightly towards the end of the experiment. This apparently indicated that secondary fermentation had occurred and this conclusion is confirmed from the gaseous loss graph (Figure 13).

Losses

The effluent and gaseous losses are recorded in Figures 12 and 13. Silo C shows the most extraordinary result in that the gaseous losses at the end of the experiment were about three times as much as from silos B and D. The gaseous loss from silo A was again very low, less than 1·0 per cent of the fresh grass ensiled.

Table 16 shows the detailed losses of individual constituents. The total percentage dry matter losses for silos A, B, C, and D were 17·4 ; 23·7 ; 34·6 and 21·6 respectively. The corresponding percentage gaseous dry matter losses were 10·0 ; 18·1 ; 26·9 and 14·1.

TABLE 16

Experiment 3

Percentage losses during ensilage

	A Total	A Effluent	B Total	B Effluent	C Total	C Effluent	D Total	D Effluent
Total	29·4	28·5	26·1	24·6	34·3	29·5	31·5	29·9
Dry matter	17·4	7·4	23·7	5·6	34·6	7·7	21·6	7·5
Crude protein	16·7	13·5	15·7	10·0	31·5	14·8	16·9	13·2
Ether extract	+20·1	—	0·4	—	+0·8	—	+6·3	—
Crude fibre	8·7	—	16·4	—	25·3	—	15·0	—
N.F.E.	28·1	—	36·2	—	44·6	—	31·8	—
Total N	16·7	13·5	15·7	10·0	31·5	14·8	16·9	13·2
Protein N	57·6	—	42·6	—	63·9	—	—	—
Total Carbohydrates incl. lignin	41·3	—	46·1	—	52·7	—	39·3	—
Sugars	87·2	19·0	94·2	8·8	91·6	9·8	92·9	10·4
Fructosan	96·8	—	96·8	—	98·2	—	96·5	—
Cellulose (C. & M.)	6·9	—	16·3	—	24·8	—	13·2	—
Cellulose (glucosan)	11·2	—	27·9	—	30·3	—	18·8	—
Hemicelluloses	41·0	—	28·6	—	50·5	—	14·4	—
Galactan	45·7	—	56·7	—	31·3	—	23·9	—
Araban	57·4	—	66·0	—	67·7	—	37·7	—
Xylan	35·6	—	13·7	—	46·2	—	5·8	—
Lignin	24·3	—	22·8	—	33·6	—	21·3	—
Water	31·5	32·2	26·6	27·9	34·3	33·3	33·3	33·8

In the previous July experiment the total dry matter loss amounted to only 7·0 per cent and it is clear that the relatively large loss in the third experiment is in part due to the larger volume of effluent produced. In spite of this the gaseous dry matter loss in Experiment 3 was more than twice that which occurred in the first experiment. Possibly an important factor in the explanation of this is that the pH of the silage, as reflected in the effluent values, fell rather more slowly in the third experiment than in the first. The very wet nature of the original herbage may also have been an important factor.

The percentage loss of dry matter from silo D was slightly lower (21·6) than that lost from silo B (23·7) and indicated that the consolidation weights applied to D on the 16th day did reduce the losses slightly.

The differences are more marked when the dry matter gaseous losses are considered. The dry matter loss from silo C (34·6) was very much greater than from B and D. These high losses are due mainly to greater breakdown of carbohydrates, particularly the cellulose, hemicelluloses and lignin.

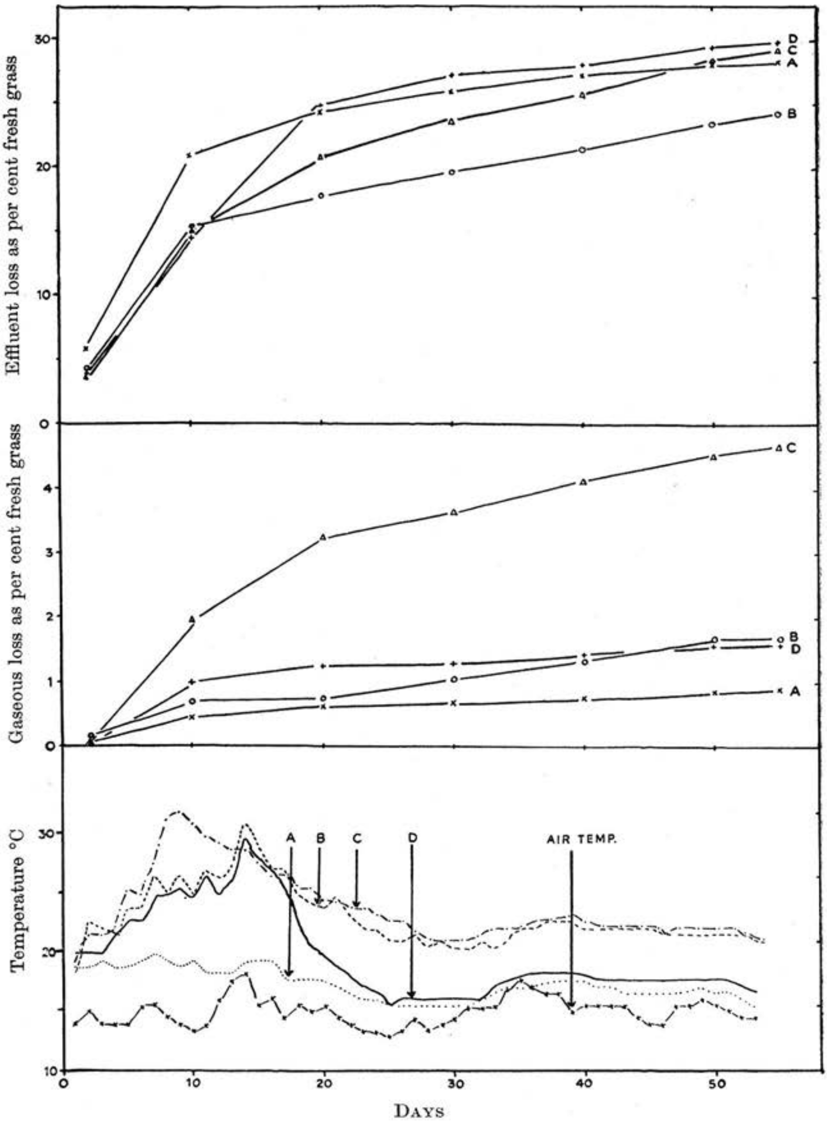

FIG. 12 (top). Weight of effluent as percentage weight fresh grass ensiled. Expt. 3
FIG. 13 (middle). Gaseous loss as percentage weight fresh grass ensiled. Expt. 3
FIG. 14. Variation in temperature (°C) changes in silages (mean of nine thermocouples). Expt. 3

TEMPERATURE CHANGES

The temperature changes within the silos were recorded daily. In silo A a fault arose shortly after the commencement of the experiment in the thermocouple wires leading from the silo and because of this, temperatures were recorded daily using maximum thermometers inserted via the ports in the top, middle and bottom layers. The mean values for silo A are therefore derived from only three daily recordings.

The maximum individual temperature recorded in silo A was 26°C on the 7th day. The highest temperature recorded in silo B was 36°C on the 14th day, *i.e.* the day immediately following the largest injection of oxygen. At the end of the ensiling period the temperature of the upper layers in silo B was still high (24°C). In silo C the highest individual temperature recorded by the thermocouples was 44°C on the 8th day. This temperature was recorded by thermocouple No. 8 which had been originally sited at the 140 cm. level, 20 cm. from the silo wall. The temperature of the central thermocouple at this level was 42°C. At the end of the experiment the temperature in the top layers of silage C was 29°C. In silo D the maximum temperature was recorded on the 14th day (37°C), *i.e.* similar to that in silo B. The effect of applying consolidation weights to silo D on the 16th day was quite dramatic in reducing the temperature. The mean temperature results are shown in Figure 14. In addition to the silage temperatures, the mean daily air temperatures, as recorded on a thermograph, are shown. It can be seen from this graph that a second peak occurs in all four silos between 30-40 days after ensiling, but this is of doubtful significance, since a similar rise in air temperature occurred at this time.

The maximum temperature was recorded in silo C; this was unexpected since this silo had not been lagged. It would appear that the Fibreglass insulation of silos B and D had little, if any, effect in preventing loss of heat via the metal walls and since B and D had received slightly more consolidation weights applied to the surface than C, this probably accounted for the lower temperatures recorded.

It is possible that higher temperatures might have occurred if the dry matter of the original herbage had been higher. In the author's opinion, however, electrothermal insulation of the silos is desirable if high temperature silages are to be produced in metal silos of this type.

It is clear from the temperature results that injection of oxygen into silos B and D did increase the temperature due to fermentation and respiration reactions. This occurred to quite a considerable extent even on the 13th day.

Nutritive value

The digestibility coefficients for the grass and silages are given in Table 17. Contrary to the findings of the previous two experiments, some differences in digestibilities between the grass and silages occurred. Generally the nutrients in the original herbage were more digestible than in the silages although the constituents in silage A, excluding the N.F.E. fraction, were almost as digestible as in the grass.

The most interesting results are seen in the protein figures and the values for two of the sheep consuming silage C were relatively low. The lowest C.P. digestibility was 53·9 which is 17 per cent lower than the lowest value obtained for sheep on silage A. One animal consuming silage C gave a digestibility coefficient not dissimilar from silage A values.

It has been reported that ' overheated ' silage tends to affect adversely the protein digestibility (Watson 2) and it is significant that the highest temperatures during the experiment were recorded in silo C. The maximum temperature recorded was only 44°C which is commonly obtained under farming conditions. It is possible that this temperature had adversely affected the protein digestibility, although the conclusion is complicated by the fact that a considerable loss of nitrogenous compounds occurred other than in the effluent. These presumably were lost as ammonia from the top layers of the high pH material. It is possible that this breakdown of nitrogenous compounds occurred mainly in the more soluble and digestible nitrogen fraction, which could account for the lower digestibility of the residual nitrogenous substances. Before concluding that temperatures of the order of 44°C can reduce protein digestibility it is clear that further work on this subject is desirable.

The digestible nutrients have been calculated and are shown in Table 17. The S.E. and T.D.N. values were lower than those in the original grass, the highest value in the silages occurred in silage A. The lowest energy values were obtained in the unweighted silages B and C. This is presumably a reflection of the higher soluble carbohydrate losses which occurred in these two silos. In calculating the S.E. values, Kellner's method, in which the ether extract (E.E.) is multiplied by 1·9, has been used. It is obvious from a consideration of the higher E.E. values obtained for the silages, that a large proportion of this fraction includes constituents, other than oils, which are of a relatively low energy value. No allowance has been made for these constituents in our calculations and because of this the S.E. and T.D.N. values will be slightly on the high side. The errors, however, are not great and are not liable to exceed one unit of the S.E. or T.D.N.

TABLE 17

EXPERIMENT 3

Percentage digestibility (D.) and percentage of digestible nutrients (D.N.)

	Grass		Silages							
			A		B		C		D	
	D.	D.N.	D.	D.N.	D.	D.N.	D.	D.N.	D.	D.N.
Dry matter	73·1	—	67·7	—	65·3	—	63·7	—	66·0	—
Organic matter	77·8	69·1	73·7	63·8	71·6	61·2	66·6	58·0	71·9	62·1
Crude protein	68·2	8·2	65·9	8·0	64·5	8·5	60·0	7·5	62·3	7·9
Ether extract	64·0	1·6	70·7	2·5	69·1	2·2	69·4	2·6	69·7	2·3
Crude fibre	81·9	21·4	79·8	23·1	80·0	22·9	78·5	23·5	80·2	22·8
N.F.E.	78·6	38·0	71·9	30·2	67·9	27·4	66·0	27·0	69·1	29·0
S.E.	—	62·4	—	57·1	—	54·2	—	53·8	—	55·3
T.D.N.	—	71·0	.	66·9	—	63·8	—	63·8	—	64·8

Many of the nitrogen balance figures in the silages (Appendix I) were again negative, the lowest occurring in silage C. The mean daily intake of D.C.P. in this group of sheep amounted to 45·8 g. or 0·1 lb./day which according to Woodman should be more than adequate for maintenance. The dry matter intake of the sheep consuming silage C averaged 366 g./day which corresponded to an intake of 197 g. of S.E. (0·431 lb.). According to Watson (27) the S.E. requirement for maintenance of sheep of this size is about 0·75 lb./day. It is clear from this that the energy content of the ration was deficient in spite of the fact that the sheep had been fed to appetite.

Because of this, it is most probable that the low negative values for nitrogen were a result of protein catabolism in the animal body.

The mean dry matter intake of the sheep consuming the fresh herbage was 739 g./day which corresponds to an intake of 60·3 g. (0·13 lb.) D.C.P. and 461 g. (1·02 lb.) of S.E. The energy content of this diet should be adequate for maintenance and the production of about ¼ lb. liveweight gain per day. According to Watson and More (26) about 0·2 lb. D.C.P. per day is required for maintenance and maximum production, therefore the protein intake was rather on the low side.

In experiments of this type it has been found in all trials that sheep are reluctant to consume as much silage as fresh grass and invariably the appetite is reduced to considerably less than the theoretical values usually quoted for sheep in this liveweight range. Although this is unlikely to affect the digestibility of individual constituents to any extent, unless silage of high nutritive value is fed, the nitrogen balance results are likely to be misleading. In this last experiment the crude protein content of the silage was considerably lower than that of the silages in the previous two experiments and this, coupled with the low energy intake, largely accounts for the low nitrogen utilisation values.

Bacteriological results

A set of laboratory silages was made with herbage similar to that used for filling the large silos and held at 30°C as in the other two experiments. Since the variation in consolidation and insulation of the large silos would result in temperature variations in the silage, further series of laboratory silages were set up and held at different temperatures. These were (*a*) held at room temperature throughout the experiment, (*b*) held at room temperature for four days and then held at 22°C for the rest of the period, (*c*) held at room temperature for one day followed by 30°C for four days, 40°C for a further twenty-eight days and 37°C until the final examination. These silages were examined after 2, 6, 12 and 72 days with the exception of the examination after 2 days in series (*b*) which was omitted because the holding temperature at that time was the same as in series (*a*).

Fresh herbage.—The ' total ' count of organisms on the fresh herbage was similar to that obtained in the previous experiment while the count of Gram-negative types on lactate agar was higher (Table 18). The number of organisms growing on acetate agar was smaller than that found in Experiment 1 but was more than ten times the number obtained in Experiment 2. All the cultures isolated on this medium proved to be heterofermentative types of streptococci or lactobacilli.

Laboratory-made silage.—The examination after 6 days did not include a count of anaerobic organisms.

Although the initial seeding with lactobacilli was slight, these bacteria multiplied with great rapidity to give a count after two days which formed most of the bacterial population in the silage held at 30°C (Figure 15). The high moisture content of the crop probably facilitated this rapid growth by making the plant nutrients readily available to the organisms. Homofermentative lactobacilli were isolated at each examination of the silage held at 30°C; heterofermentative streptococci were isolated only at the examination after two days.

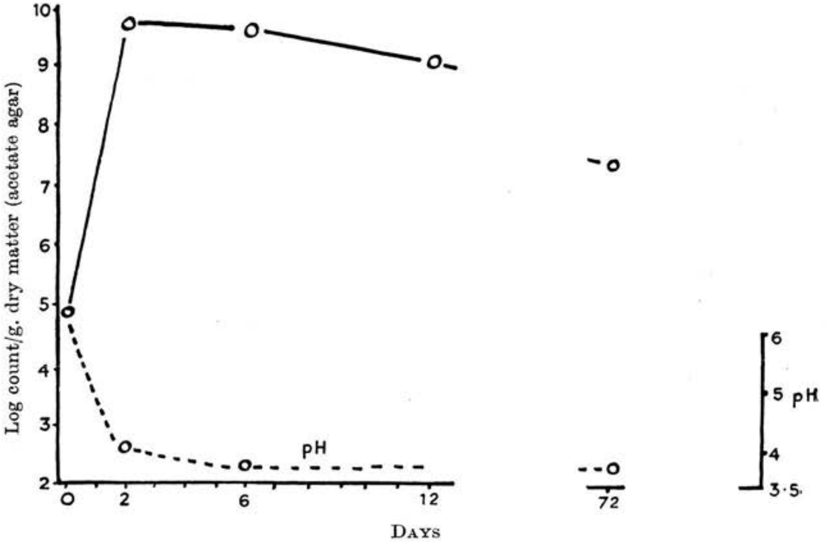

FIG. 15. Lactobacillus counts and pH values obtained from laboratory-made silage held at 30°C

In the silage held at room temperature during the entire period the count on acetate agar was much lower in the early stages than in silage held at the higher temperature.

Heterofermentative streptococci were numerous while homofermentative lactobacilli were isolated less frequently than the heterofermentative type. Much the same result was obtained when a holding temperature of 22°C followed room temperature. A decrease in bacterial numbers resulted when the silage was placed at 40°C and streptococci which were present at two days appeared to be replaced by lactobacilli by twelve days. There were very few bacteria in the high temperature silage by seventy-two days. The selective action of temperature on the microflora of silage has recently been discussed (Gibson *et al.* 28).

TABLE 18

The pH values and bacterial counts from grass and silage in Experiment 3

	Bacterial count (millions/g. dry wt. grass) on			of	
	Glucose yeast agar	Acetate agar	Lactate agar	Anaerobes	pH
Fresh grass	590	0·1	160	<0·01	—
Laboratory silage					
30°C—2 days	5900	5000	<0·04	<0·01	4·2
6 ,,	4700	4200	<0·01	—	3·8
12 ,,	850	1000	<0·01	0·03	—
72 ,,	30	30	<0·01	7·3	3·8
Room temperature					
2 days	5000	1200	<0·04	<0·01	4·5
6 ,,	4400	3100	0·26	—	4·3
12 ,,	3800	3800	<0·01	<0·01	—
72 ,,	110	105	<0·01	<0·01	3·7
Room temperature + 22°C					
2 days	—	—	—	—	—
6 ,,	3600	3800	0·02	—	4·2
12 ,,	4300	3600	<0·01	<0·01	—
72 ,,	45	45	<0·01	<0·01	3·7
Room temp. + 30°C, 40°C, 37°C					
2 days	5500	3000	<0·04	<0·01	4·3
6 ,,	620	620	<0·01	—	3·9
12 ,,	85	81	<0·01	<0·01	—
72 ,,	0·04	0·04	<0·01	<0·01	3·9
Experimental silage					
Silo A	890	950	2·3	0·03	3·7
,, B	3800	4200	46	3·3	3·9
,, C	3200	4500	110	16	4·1
,, D	2000	2700	3·8	3·3	3·8

Experimental silages.—Silage A gave the lowest pH value. The bacterial counts of this silage were of the same order as those obtained in the previous experiments and the count of anaerobes was low. The bacterial counts from the other silages were higher than previously; silage C gave the highest pH and the highest count of anaerobes. Pediococci and both homofermentative and heterofermentative lactobacilli were isolated.

Lactic acid bacteria.—A point of interest in this experiment was the dominance of heterofermentative streptococci among the relatively few lactic acid bacteria that were present on the fresh herbage. In spite of the initial scarcity of lactobacilli, both homofermentative and heterofermentative types developed rapidly and later superseded the streptococci. The pH values of the laboratory-made silages decreased less rapidly at the lower temperatures than when the silage was held at 30°C or higher, but at the examination after seventy-two days they were slightly lower.

GENERAL DISCUSSION

It has been shown in the first experiment that it is possible to produce well preserved silage of similar nutritive value to that of the original grass with dry matter losses as low as 7 per cent and no 'waste' material. The results show that similar silages can be produced in separate silos provided uniform consolidation is obtained. In the second and third experiments, silo A was filled as in the first experiment but the other silos received different consolidation treatments. The effect of varying the consolidation weight applied to the surface of the herbage was mainly on the quantity of 'spoiled' material produced in the top layers of the silage. The results of the second experiment show that well preserved silage can be produced in the bottom layers of a tower silo which has not had consolidation weights applied, but undesirable fermentation, including mould growth, is likely to produce inedible material to a depth of about 12 cm. In the second experiment, this corresponded to 20 per cent of the original weight of herbage ensiled. It is clear from these experiments that with adequate consolidation, *i.e.* a pressure of about 37 g./sq. cm. (76 lb./sq. ft.), the top spoilage can be reduced to a minimum. If containers, other than towers, are used it is obvious that precautions against the production of side wastage are also necessary.

The herbage used in Experiment 1 was similar in chemical composition to that used in Experiment 2. The 'available' carbohydrate content of the ensiled grass was 15·1 per cent in Experiment 1 and 16·1 per cent in Experiment 2. The dry matter values were also of the same order. In spite of this the control silos (A) showed quite different results. The pH values of the silage produced from summer and autumn grasses were 3·8 and 4·3 respectively. The dry matter losses amounted to 7 per cent and 17·4 per cent respectively.

Although the chemical composition of the herbage used in the two experiments did not differ greatly, it differed considerably in its content of lactobacilli. These organisms were present in appreciable number on the grass used in Experiment 1, but were scarce on that used in Experiment 2. The acid-producing power of the lactobacilli makes them important for promoting a low pH in silage, so it might have been expected that the amount of carbohydrate used and the quantity of lactic acid formed would have been greater in Experiment 1 than in the second experiment. A comparison of the results obtained from silo A in the two experiments, however, shows that the loss of carbohydrate was almost the same in each fermentation.

Examination of the silages made in the laboratory showed that in the first experiment lactobacilli were dominant within 3 days and by that time the pH had fallen to 4·2. In contrast, after 5 days in the second experiment the pH was not below 5·8 and lactobacilli comprised less than one-tenth of the bacterial population. Thus in this second experiment other organisms such as the Gram-negative types and the anaerobes assumed greater importance in the early stages of the fermentation. The action of these organisms might provide some explanation for the greater losses in Experiment 2. Not only are the products of fermentation more varied, including acetic acid, carbon dioxide and hydrogen, but decarboxylation of amino acids and the formation of amines and ammonia would tend to keep the pH at a higher level.

In the second experiment the high gaseous losses from silo A (13 per cent) compared with losses from this silo (4·4 per cent) in the first experiment have been attributed to a difference in the fermentation due to the lack of lactobacilli on the original grass ensiled. It is usually considered that carbohydrates are the main compounds responsible for gaseous loss, but since the total carbohydrate breakdown in the experiments was approximately the same, it is clear that there must have been other undetermined compounds in the N.F.E. fraction which played an important part in the changes which occurred. Considerable carbohydrate breakdown during ensilage occurs with the production of compounds other than lactic and volatile acids. The nature of some of these compounds has already been referred to in a preceding section.

Although the apparent gaseous losses have been calculated in these experiments, no allowance has been made for the gaseous oxygen used in aerobic changes and because of this, it is impossible to relate these gaseous losses to carbohydrate changes. It is obvious that the many reactions which occur are very complex and the only true way of assessing the quantities of gaseous products is by collection from sealed containers.

The pH value of the silage produced from silo A in the second experiment was 4·3 compared with a value of 3·8 for the same silo in the first experiment. Lactic acid is mainly responsible for the acidity of silage (Watson 2) and yet the quantities of this acid produced in Experiments 1 and 2 were 14·04 and 15·56 kg. respectively. The total amounts of dry matter obtained from the silos were 162·1 and 149·3 kg. It is difficult to relate these figures to the resultant pH values. A number of factors could have been responsible for the differences. Firstly the quantity of volatile nitrogenous compounds produced in the second experiment was higher than in the first experiment. These compounds would presumably play a part in neutralising the lactic acid. Secondly the extent of protein breakdown is likely to affect the buffering nature of the silage and because of this effect it is difficult to relate pH to lactic acid concentration. Furthermore it is known that a number of bacteria present in silage are capable of decarboxylating amino acids. This reaction can also be brought about by plant decarboxylases. It has been shown that a large part of gamma-amino butyric acid, the decarboxylated residue from glutamic acid, can be formed by these plant enzymes (Macpherson and Slater 29). The resultant basic residues are themselves capable of neutralising the acids in the silage mass. Unfortunately in these studies, apart from volatile nitrogen determinations, no detailed investigations were made of the nature of the non-protein nitrogenous compounds.

The results obtained in all three experiments indicate that considerable carbohydrate breakdown occurred other than sugars and fructosan. This occurs even in well preserved silage where the dry matter losses are low and under these conditions it is mainly the hemicelluloses which are affected to any extent.

Harwood (17) has studied the changes in cell wall polysaccharides during ensilage of perennial ryegrass and obtained losses of 35 per cent araban and 26 to 58 per cent xylan. He concluded that in the absence of sufficient water-soluble carbohydrates lactobacilli survive by attacking araban and xylan. This action occurred in silage near the neutral point whereas in acidic silages hydrolysis of araban and xylan was the destructive mechanism.

According to Orla-Jensen (30) pentosans are probably fermented by *Betabacterium pentoaceticum* in old silage. de Man (31) showed that *Streptobacterium casei* was able to ferment galactan in potato pulp sterilised previously with ethylene oxide.

Macpherson, Wylam and Ramstad (32) have studied the carbohydrate changes during ensilage and have shown that silage preserved with metabisulphite contained at the end of a 175 day ensiling period considerably more total water-soluble sugar than the original grass. It was suggested that the increase in sugars had been brought about by hydrolysis of insoluble cell-wall polysaccharides and it appeared that about 6 per cent of cellulose had been degraded in some way. The presence of free pentose sugars in the silage extracts indicated that pentosans had been broken down.

Wylam (33) has found that hydrolysis of grass polysaccharides occurred during ensilage and has shown it to be partly due to chemical hydrolysis by acid produced in silage, particularly in the case of the labile fructosan molecules. The existence of enzymes responsible for these reactions was confirmed by the isolation from perennial ryegrass of an active cell-free extract which hydrolysed both sucrose and the fructosan isolated from ryegrass. This preparation was also found to hydrolyse a hemicellulose mixture isolated from ryegrass, with production of xylose, arabinose and glucose. Maximum hydrolysis of both fructosan and hemicellulose was effected at pH 5-6, but considerable decomposition also occurred at lower pH values.

Two strains of *Lactobacillus* and several other organisms which occur in silage were examined by Wylam for polysaccharide-hydrolysing activity. Whereas cell suspensions of one strain of *Lactobacillus* were inactive, suspension of another readily hydrolysed ryegrass fructosan with production of fructose, glucose and oligosaccharides, as well as effecting fermentation to lactic acid. Neither of these had any effect on ryegrass hemicelluloses. From a hemicellulose preparation *B. pumilus* and *B. licheniformis* produced traces of xylose and oligosaccharides, *B. polymyxa* also produced some arabinose and glucose. The results indicated that three factors were responsible for polysaccharide breakdown in silage, namely chemical hydrolysis and the action of plant and bacterial enzymes. Chemical hydrolysis would increase as the pH decreased. Since grass invertases and hemicellulases were most active at pH 5-6 they probably played a large part in hydrolysis during the initial stages of ensilage. Bacterial hydrolysis of fructosan was proportionately greatest at low pH, while hydrolysis by anaerobic bacteria might be responsible for hemicellulose decomposition in bad high pH silage.

It is difficult to say to what extent hemicellulose breakdown products are important in fermentation reactions during ensilage and it is clear that further investigation is required.

Considerable proteolysis occurred in all silage experiments and this can be regarded as a normal process during ensilage, being due almost entirely to proteolytic enzyme action (Macpherson 34). The results of nitrogen balance experiments with lambs indicate that the nitrogenous compounds may not be as well utilised in silage as in fresh herbage; it has been shown in previous trials that the addition of soluble sugars to silage at the time of feeding can increase considerably the utilisation of total N (McDonald 35). The combined effects of high soluble N with low available sugars may be a significant disadvantage under feeding systems where large quantities of silage are consumed and where a relatively large supply of protein to the animal may be required. The results of nitrogen balance trials, however, can be misleading especially where the dry matter intakes are abnormally low and it has been shown in Experiment 3 that protein catabolism due to low energy diets can falsify the results.

The effluent production from a silo is related to the original moisture content of the ensiled crop and to the consolidation weight applied. This can be seen from the results recorded in Experiment 2. It is obvious, however, from a comparison of the results obtained for silo A in Experiments 1 and 2 that other factors apart from these influence effluent flow. In spite of the similar treatments applied to silo A in both experiments and the similar moisture contents of the original herbage ensiled, about 50 per cent more effluent was recovered from this silo in the second experiment than in the first over a similar period of time. The high gaseous loss in the second experiment must be associated with this result. It is natural to conclude that the higher gaseous loss was due mainly to respiration changes, and presumably the water produced from carbohydrate breakdown would contribute to the larger effluent flow but in addition a decrease in carbohydrate content automatically increases the percentages of the other constituents, including moisture. Because of this, the larger the dry matter loss due to respiration, the greater will be the moisture percentage of the resultant silage. It is clear from this that the effluent flow will also be related to the gaseous losses and, therefore, the higher the dry matter losses during ensilage, the greater the effluent production. This fact is verified in Experiment 3 where in the two unweighted silos, B and C, the latter produced considerably more effluent than B as well as higher dry matter losses.

It is obvious that the production of high temperature silage results not only in higher respiration losses, but also in a greater effluent flow which is undesirable. These experiments have shown that where conditions are ideal, low temperature silage can be made with extremely low losses, the final product having a nutritive value similar to that of the original herbage. Under farming conditions, however, the system of allowing the herbage to heat up to a temperature of 27°-33°C during filling is still the general practice in this country as an insurance against undesirable fermentation.

Although the effluent production in silo A of the third experiment amounted to 259 kg. compared with 59 kg. from the same silo in Experiment 1, the corresponding dry matter loss via this source amounted to 10 kg. and 4·5 kg. respectively. The higher value represents a dry matter loss of 7·4 per cent which, despite the large volume of effluent produced, cannot be considered as excessive. When individual constituents are considered, however, the disadvantages of a large volume of effluent become more apparent. In Experiment 1 only 1·9 per cent of the sugars were lost via this source whereas in Experiment 3 the loss of sugars amounted to 19 per cent. These percentage calculations are based on the original weight of sugar added but it is clear that some of the effluent sugars included in these figures have come from hemicellulose breakdown. Reference to the effluent composition figures for Experiment 3 in Appendix I, however, indicates that the greater proportion of sugars was lost in the early stage of ensilage and it is unlikely that breakdown of hemicelluloses contributed greatly at this stage to the sugar value. In view of this the ensilage of a wet crop, with consequent reduction in soluble sugar content due to heavy effluent flow in the early stages of ensilage, may be detrimental to the subsequent lactic acid production.

The results of digestibility trials with sheep have shown that the nutritive value of silages may be very similar to that of the original herbage. Since the water soluble nutrients in silages are generally the most digestible, it follows that under conditions where the effluent production is great then the nutritive value is likely to be correspondingly low. The ensiling of a wet crop, as in Experiment 3, will contribute to these conditions and the relatively low S.E. value of silage A in this experiment is a reflection of this.

It is well known that high temperatures in silage are likely to affect adversely the protein digestibility. In silo C of Experiment 3 a maximum temperature of 44°C was obtained. It is significant that the protein digestibility of the resultant silage was considerably lower than in silo A in which the maximum temperature reached was only 26°C. It is difficult, however, to reach any definite conclusion as to the effects of the ' high ' temperature produced in silo C since a considerable amount of nitrogen (16·7 per cent) was lost in a gaseous form and it is possible that the resultant nitrogenous constituents were of lower digestibility. It seems, however, that high temperature conditions are not desirable.

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APPENDIX I

TABLE 19

Composition of effluent from silo A. Expt. 1
(mg./g.)

Day	Total N	Vol. N	Amide N	Glutamine N	Acetic acid	Dry matter	Ash	Red. sugars	Total sugars	pH
3	1·87	·12	·10	·02	1·01	52·7	15·6	6·9	13·4	4·4
4	2·53	·16	·12	·03	1·34	61·2	16·3	6·8	8·4	4·3
5	2·91	·21	·12	·02	1·50	64·4	16·4	7·8	8·2	4·2
6	3·01	·23	·12	·03	1·67	66·6	16·3	9·8	13·6	4·2
7	3·20	·24	·12	·02	1·78	67·0	16·8	8·6	—	4·1
8	3·15	·25	·18	·03	1·82	68·0	17·0	7·1	6·8	4·2
9	3·27	·27	·14	·02	1·93	69·7	17·1	7·3	8·1	4·2
10	3·31	·27	·14	·04	2·18	70·0	16·8	7·4	7·9	4·1
13	3·21	—	—	—	2·23	69·3	18·5	—	—	—
18	3·68	·33	·13	·02	2·75	75·6	16·9	5·9	7·1	4·0
21	3·69	·36	·10	—	3·12	79·8	20·1	3·2	—	4·2
25	3·64	·39	·12	—	3·67	81·3	20·9	2·3	—	4·3
26	3·91	·39	·06	—	3·28	80·7	17·6	4·8	5·3	4·1
29	3·92	·39	—	—	3·64	82·4	20·0	3·3	—	4·2
32	4·04	·40	·12	—	3·68	82·2	18·3	3·9	4·6	4·1
37	4·19	·40	·15	—	4·05	85·2	18·0	2·9	3·5	4·0
44	4·26	·59	—	—	4·32	84·2	17·9	3·9	—	4·0
51	4·40	·49	—	—	4·56	88·1	18·2	3·7	—	4·0
58	4·45	·54	—	—	4·52	85·1	17·9	4·1	—	—
63	4·45	·51	—	—	4·67	89·0	18·6	3·3	—	—

TABLE 20

Composition of effluent from silo B. Expt. 1
(mg./g.)

Day	Total N	Vol. N	Amide N	Glutamine N	Acetic acid	Dry matter	Ash	Red. sugars	Total sugars	pH
3	2·07	·12	·09	·02	1·01	57·5	16·2	8·2	11·2	4·4
4	2·54	·16	·11	·03	1·30	62·2	16·5	7·8	12·0	4·3
5	2·85	·20	·12	·02	1·56	65·7	16·7	8·1	7·6	4·2
6	3·03	·22	·13	·02	1·69	67·9	16·7	10·5	12·0	4·2
7	3·20	·24	·15	·02	1·75	69·8	17·0	8·8	9·7	4·2
8	3·27	·26	·13	·02	1·84	69·1	17·0	8·6	8·3	4·1
9	3·32	·26	·17	·02	1·94	71·7	17·2	8·3	9·7	4·2
10	3·30	·27	·14	·03	2·05	72·2	17·1	8·5	9·6	4·2
11	3·43	·28	·16	·02	2·16	71·9	17·5	8·3	9·0	4·2
13	3·47	·29	·14	·02	2·36	73·6	19·1	7·2	7·4	4·2
17	3·70	·32	·14	—	2·51	76·6	16·9	6·8	7·3	4·0
20	3·82	·34	·13	—	2·90	79·2	17·7	6·5	7·4	4·1
23	3·74	·38	·10	—	3·28	80·7	20·0	3·9	—	4·3
25	3·88	·39	·12	—	3·68	81·5	19·7	4·5	—	4·3
26	3·94	·38	·12	—	3·26	83·6	19·6	4·8	5·3	4·2
29	4·07	·39	·13	—	3·67	84·0	19·9	3·8	—	4·2
32	4·07	·39	·13	—	3·67	82·2	18·7	4·4	4·9	4·1
37	4·16	·41	·13	—	4·18	84·3	18·7	3·4	4·5	4·1
44	4·27	·50	—	—	4·35	83·1	18·2	3·1	—	4·0
51	4·37	·46	—	—	4·53	88·0	18·1	3·6	—	4·0
58	4·52	·53	—	—	4·95	84·5	17·6	3·6	—	—
63	4·47	·49	—	—	4·78	90·1	17·7	3·6	—	—

TABLE 21

Composition of effluent from silo C. Expt. 1
(mg./g.)

Day	Total N	Vol. N	Amide N	Glutamine N	Acetic acid	Dry matter	Ash	Red. sugars	Total sugars	pH
3	2·28	0·13	0·13	0·03	1·18	58·0	16·3	7·4	11·2	4·3
4	2·60	0·17	0·12	0·03	1·37	62·7	16·2	7·7	18·1	4·2
5	2·95	0·21	0·13	0·03	1·53	66·5	16·2	7·2	9·2	4·2
6	3·10	0·24	0·14	0·01	1·78	68·8	16·6	10·6	—	4·1
7	3·04	0·23	0·13	0·03	1·80	66·5	17·1	9·0	8·5	4·1
8	2·23	0·27	0·14	0·03	2·00	69·4	16·9	8·7	8·5	4·1
9	3·40	0·27	0·18	0·03	2·17	72·3	16·9	7·7	9·2	4·1
10	3·43	0·27	0·14	0·04	2·21	73·0	16·9	8·0	8·8	4·1
13	3·61	0·30	0·13	—	2·26	75·2	18·7	6·2	7·1	4·2
17	3·60	0·31	0·16	—	2·93	79·4	19·7	4·4	5·6	4·2
18	3·83	0·33	0·14	—	2·85	78·8	18·3	6·7	—	4·1
19	3·85	0·36	0·13	—	2·89	78·9	18·4	6·1	—	4·1
20	3·96	0·35	0·13	—	3·02	80·7	18·8	5·7	6·1	4·1
21	3·91	0·37	0·13	—	3·17	81·7	19·0	4·8	—	4·2
23	3·85	0·38	0·11	—	3·23	81·3	18·3	4·9	—	4·1
25	4·04	0·40	0·14	—	3·47	83·7	19·1	4·2	—	4·1
27	4·07	—	—	—	3·46	82·8	18·8	4·1	4·8	4·0
29	4·22	0·40	0·13	—	3·66	85·6	19·2	3·9	—	4·0
32	4·16	0·40	0·12	—	3·92	84·1	19·1	3·5	4·0	4·0
37	4·23	0·42	0·13	—	4·14	86·5	19·1	3·5	4·2	4·0
44	4·30	0·44	—	—	4·50	85·5	18·2	3·7	—	3·9
51	4·41	0·45	—	—	4·86	89·0	19·0	—	—	4·0
58	4·40	0·46	—	—	4·66	84·1	17·8	3·7	—	—
63	4·53	0·47	—	—	4·64	91·0	18·7	3·9	—	—

TABLE 22

Composition of effluent from silo D. Expt. 1
(mg./g.)

Day	Total N	Vol. N	Amide N	Glutamine N	Acetic acid	Dry matter	Ash	Red. sugars	Total sugars	pH
4	2·37	0·15	0·10	0·03	1·15	60·8	16·8	7·9	1·21	4·3
5	2·84	0·20	0·14	0·03	1·59	67·0	16·8	8·4	8·3	4·2
6	2·98	0·22	0·14	0·02	1·56	67·8	16·5	10·8	—	4·1
7	3·10	0·23	0·13	0·03	1·75	68·6	16·6	9·7	—	4·1
8	3·22	0·25	0·14	0·02	1·82	68·6	17·1	9·3	—	4·2
9	3·30	0·24	0·12	0·02	1·87	71·2	16·9	9·0	10·1	4·2
10	3·34	0·26	0·13	0·03	2·03	72·4	17·5	8·8	10·2	4·2
11	3·38	0·27	0·15	0·02	2·02	72·4	18·2	8·5	8·6	4·2
13	3·41	0·28	0·12	—	2·26	72·3	18·5	7·6	7·0	4·2
17	3·47	0·32	—	—	2·74	78·5	19·9	6·7	—	4·2
20	3·49	0·34	0·13	—	3·21	78·7	20·9	3·7	4·2	4·3
21	3·72	0·34	0·13	—	2·93	78·9	17·7	7·2	—	4·1
23	3·99	0·35	—	—	3·07	79·4	17·8	6·6	—	4·1
25	3·81	0·37	0·12	—	3·12	80·3	18·1	6·5	—	4·1
27	3·94	—	—	—	3·31	81·5	17·7	6·4	6·6	4·1
29	4·05	0·37	0·13	—	3·45	82·7	17·3	5·8	—	4·0
32	4·09	0·38	0·12	—	3·53	82·2	17·4	5·9	6·0	4·0
37	4·06	0·39	0·13	—	3·84	83·3	17·1	3·8	5·8	4·0
44	4·23	0·49	—	—	4·20	84·6	17·9	4·3	—	4·0
51	4·32	0·45	—	—	4·54	87·4	18·6	4·0	—	4·0
58	4·49	0·45	—	—	4·67	82·6	18·1	3·6	—	—
63	4·36	0·54	—	—	4·65	89·0	18·2	3·9	—	—

TABLE 23

Composition of effluent from silo A. Expt. 2
(mg./g.)

Days	Total N	Vol. N	Amide N	Acetic Acid	Dry matter	Ash	Red. sugars	Total sugars	pH
1- 7	2·21	0·12	0·08	—	51·4	15·1	11·2	—	5·9
8- 14	3·20	0·30	0·10	0·98	63·0	16·5	8·7	—	4·4
15- 21	3·85	0·38	0·11	1·54	71·1	16·8	7·9	10·1	4·3
22- 28	3·97	0·41	0·11	1·52	71·4	16·8	10·3	10·2	4·3
29- 35	4·09	0·46	0·08	1·83	73·5	16·9	8·2	9·1	4·3
36- 42	4·22	0·46	0·10	2·02	74·8	16·9	—	7·3	4·3
43- 49	4·29	0·48	0·11	2·21	77·2	17·1	8·3	9·7	4·3
50- 56	4·33	0·49	—	2·44	77·7	17·0	—	9·4	4·3
57- 63	4·38	—	—	2·65	79·8	16·8	7·4	—	4·2
64- 70	4·42	—	—	2·88	81·3	16·8	—	—	4·2
71- 77	4·49	—	—	2·94	83·1	17·0	8·3	—	4·2
78- 91	4·52	—	—	3·07	82·7	17·2	7·7	—	—
92-105	4·61	—	—	3·27	85·0	17·2	—	—	4·1
106-119	4·59	—	—	3·34	84·2	17·3	—	—	4·2
120-133	4·66	—	—	3·57	85·9	17·3	—	—	—
134-143	4·71	0·63	0·05	3·91	92·1	17·4	—	—	4·2

TABLE 24

Composition of effluent from silo B. Expt. 2
(mg./g.)

Days	Total N	Vol. N	Amide N	Acetic acid	Dry matter	Ash	Red. sugars	Total sugars	pH
1- 7	2·46	0·15	0·08	—	56·5	15·0	13·0	—	6·0
8- 14	3·36	0·33	0·08	1·00	65·4	16·1	10·9	—	4·4
15- 21	3·91	0·39	0·11	1·55	72·8	16·2	10·1	12·1	4·2
22- 28	4·10	0·50	0·10	1·73	75·7	16·8	13·0	13·0	4·3
29- 35	4·11	0·49	0·07	1·88	75·5	16·6	10·7	13·0	4·2
36- 42	4·20	0·49	0·10	1·92	75·8	16·6	—	10·3	4·3
43- 49	4·29	0·49	0·1[illegible]	2·08	77·9	16·6	11·1	9·7	4·2
50- 56	4·33	0·50	—	2·29	78·8	16·6	—	9·7	4·2
57- 63	4·36	—	—	2·67	80·1	16·6	9·7	—	4·2
64- 70	4·36	—	—	2·58	80·9	16·5	—	—	4·2
71- 77	4·39	—	—	2·77	81·4	16·8	9·0	—	4·2
78- 91	4·44	—	—	2·83	81·3	17·0	8·5	—	—
92-105	4·45	—	—	2·88	82·5	16·8	—	—	4·1
106-119	4·47	—	—	3·13	82·4	16·7	—	—	4·2
120-133	4·51	—	—	3·25	82·7	16·7	—	—	—
134-143	4·55	0·58	0·05	3·37	87·4	16·7	—	—	4·2

TABLE 25

Composition of effluent from silo C. Expt. 2
(mg./g.)

Days	Total N	Vol. N	Amide N	Acetic acid	Dry matter	Ash	Red. sugars	Total sugars	pH
1- 7	2·41	0·15	0·07	—	54·7	15·1	11·6	—	6·1
8- 14	3·42	0·34	0·07	1·06	65·7	16·3	9·7	12·2	4·5
15- 21	3·97	0·34	0·10	1·52	71·3	16·6	8·6	9·0	4·3
22- 28	4·14	0·43	0·10	1·56	74·1	16·7	11·0	11·8	4·3
29- 35	4·19	0·49	0·07	1·84	74·9	16·8	10·0	11·6	4·3
36- 42	4·26	0·49	0·09	1·88	76·2	16·7	—	9·4	4·3
43- 49	4·32	0·49	0·10	2·30	77·5	16·8	—	—	4·3
50- 56	4·34	0·49	—	2·38	78·2	16·8	—	7·8	4·3
57- 63	4·41	—	—	2·47	79·9	16·7	9·4	—	4·2
64- 70	4·41	—	—	2·55	79·8	16·7	—	—	4·3
71- 77	4·42	—	—	2·60	80·9	16·8	8·9	—	4·2
78- 91	4·44	—	—	2·86	82·1	17·0	8·7	—	—
92-105	4·46	—	—	2·90	83·3	16·9	—	—	4·2
106-119	4·50	—	—	2·92	82·7	17·0	—	—	4·2
120-133	4·53	—	—	3·18	82·8	17·1	—	—	—
134-143	4·59	0·59	0·09	3·35	88·7	17·1	—	—	4·3

TABLE 26

Composition of effluent from silo D. Expt. 2
(mg./g.)

Days	Total N	Vol. N	Amide N	Acetic acid	Dry matter	Ash	Red. sugars	Total sugars	pH
1- 7	2·54	0·13	0·08	—	55·8	15·0	12·6	—	6·5
8- 14	3·23	0·31	0·09	1·02	63·1	16·1	9·5	11·8	4·5
15- 21	3·71	0·37	0·07	1·53	68·7	16·3	9·0	10·8	4·3
22- 28	3·89	0·42	0·10	1·49	70·7	16·4	—	10·3	4·3
29- 35	3·97	0·43	0·10	1·69	71·1	16·5	8·6	11·1	4·3
36- 42	4·02	0·45	0·09	1·81	72·4	16·3	10·1	9·4	4·3
43- 49	4·10	0·47	0·10	1·77	73·7	16·3	9·8	—	4·3
50- 56	4·16	0·46	—	1·92	73·4	16·3	—	11·3	4·2
57- 63	4·19	—	—	2·00	74·0	16·2	7·4	—	4·2
64- 70	4·20	—	—	2·07	74·7	16·1	—	—	4·2
71- 77	4·24	—	—	2·11	76·2	16·5	9·4	—	4·2
78- 91	4·30	—	—	2·13	76·6	16·5	8·4	—	—
92-105	4·30	—	—	2·19	77·8	16·2	—	—	4·2
106-119	4·34	—	—	2·20	77·4	16·5	—	—	4·2
120-133	4·31	—	—	2·43	76·4	16·3	—	—	—
134-143	4·41	0·55	0·09	2·53	81·3	16·5	—	—	4·3

TABLE 27

Composition of effluents in Expt. 3
(mg./g.)

Silo	Days	Total N	Acetic acid	Butyric acid	Dry matter	Ash	Red. sugars	Total sugars	pH
A	1- 7	1·00	0·96	Nil	31·9	8·1	5·89	9·39	4·6
	8-14	1·78	2·56	,,	46·9	10·7	3·86	4·92	4·2
	15-21	2·03	3·28	,,	53·5	10·9	1·20	2·34	3·7
	22-28	2·18	3·91	,,	56·6	10·9	1·53	2·80	3·5
	29-35	2·23	4·03	,,	58·3	11·5	1·12	2·96	3·4
	36-42	2·25	4·05	,,	59·4	11·8	1·00	2·59	3·4
	43-49	2·33	4·24	,,	60·0	11·7	1·13	2·38	3·4
	50-56	2·34	4·04	,,	61·8	11·9	1·19	2·75	3·4
B	1- 7	0·88	0·92	Nil	25·7	6·9	3·70	5·62	4·4
	8-14	1·39	2·54	,,	41·1	10·5	2·09	3·13	4·0
	15-21	1·46	3·05	,,	42·1	10·6	0·58	1·62	3·7
	22-28	1·46	3·35	,,	45·4	11·0	0·61	1·49	3·5
	29-35	1·59	3·78	,,	47·0	11·4	0·53	1·34	3·4
	36-42	1·59	3·93	0·25	45·5	11·6	0·41	1·27	3·4
	43-49	1·62	4·41	0·41	47·7	11·4	0·39	1·22	3·4
	50-56	1·63	4·48	1·13	47·5	11·1	0·41	1·22	3·4
C	1- 7	0·84	0·90	Nil	27·1	7·3	3·43	5·55	4·5
	8-14	1·82	2·67	,,	46·2	11·3	2·72	3·94	4·0
	15-21	1·98	3·18	0·64	46·9	10·9	0·68	2·02	3·8
	22-28	1·84	3·18	1·32	46·4	11·0	0·82	1·81	3·7
	29-35	1·89	3·39	1·48	48·3	11·4	0·77	1·64	3·6
	36-42	1·86	3·60	2·10	47·0	11·5	0·55	1·47	3·6
	43-49	1·90	3·98	2·54	47·8	11·2	0·55	1·21	3·6
	50-56	1·83	3·79	3·32	47·0	11·2	0·60	1·29	3·7
D	1- 7	0·87	0·88	Nil	27·7	7·6	3·31	6·05	4·5
	8-14	1·46	2·46	,,	41·5	11·0	1·78	3·22	4·1
	15-21	1·61	3·20	,,	44·8	11·3	0·65	2·04	3·7
	22-28	1·59	3·58	,,	47·7	11·6	0·58	1·53	3·6
	29-35	1·63	3·72	,,	48·3	11·9	0·51	1·55	3·5
	36-42	1·68	4·05	Tr.	49·9	12·5	0·38	1·37	3·5
	43-49	1·74	4·28	,,	51·5	12·3	0·37	1·17	3·4
	50-56	1·75	4·36	,,	49·0	12·0	0·42	1·33	3·5

TABLE 28

Summary of nitrogen balance results
(Values expressed as N retained per cent total N digested)

		†Silages			
Experiment	*Grass	A	B	C	D
1	22·5	1·0	4·1	3·4	11·6
2	19·8	10·2	18·3	17·5	19·1
3	21·3	— 4·4	7·4	— 25·7	— 3·4

* Mean of four trials. † Mean of three trials.

APPENDIX II

Chemical Analyses

1. *Carbohydrates*

NOTES

1. Concentration of all solutions was carried out at 35°C under reduced pressure.
2. The chromatographic separation of sugar mixtures was carried out using Whatman No. 1 paper in a suitable solvent for 40 hrs. at 23°C. The solvents used were :—

   Solvent I. Ethyl Acetate–Acetic Acid–Water (3–1–3) (upper layer).
   Solvent II. Benzene–Butanol–Pyridine–Water (1–5–3–3) (upper layer).

   In the former, glucose and galactose travel together but may be separated in the latter. Likewise fructose and arabinose travel together in the latter, but may be separated in the former.

   In all cases the papers were sprayed with a saturated solution of aniline oxalate and heated for 3 mins. at 160°C (36).
3. Quantitative estimations were carried out by using guide strips on both sides of the paper, spraying these to show the position of the different sugars, and then cutting out the desired bands from the main part of the paper. The sugars were eluted from these bands by suspending them over boiling solvent. Efficient elution took about 1 hour.
4. Reducing sugars were estimated by the Somogyi method (37) which necessitates paper blanks adhering simultaneously to the following conditions :—

   (*a*) The chromatographic solvent used.
   (*b*) The elution solvent used.
   (*c*) Each batch of Somogyi tubes boiled together.

Grass

Sampling.—At the time of ensiling two random samples were taken ; a 20 g. sample which was immediately immersed in boiling ethanol to inhibit enzymic action (38), and a 50 g. sample which was taken back to the laboratory on which the dry matter content was estimated by heating overnight in an oven.

Free sugars and oligosaccharides.—(39) After decanting off the ethanol, and storing it, the immersed sample was macerated in fresh ethanol, transferred to a Soxhlet thimble and extracted for eight hours over 80 per cent ethanol. These ethanol extracts were combined and made up to a specific volume (ca. 1 litre). An aliquot equivalent to 1 g. dry grass was taken and xylose (ca. 20 mg.) added as a reference standard. The ethanol was evaporated off, 100 ml. water added, and the solution clarified by the addition of equimolecular volumes of cadmium sulphate and barium hydroxide at 90°C (40). The solution was cooled and filtered, the filtrate being electrodialysed between 'Permutit' ion-exchange membranes 'Permaplex C-20' and 'A-20.' The solution was then reduced to small volume and chromatogram papers run in solvent I. The glucose, fructose, and xylose were eluted with water and estimated by the Somogyi method (37). The sucrose was eluted with 1 per cent oxalic acid, neutralised with 4N sodium hydroxide, and the mixture of reducing sugars estimated by the same method. The oligosaccharides were eluted with water and then made 0·5N with sulphuric acid and hydrolysed by heating on a boiling water bath for four hours. The resultant mixture of reducing sugars, after neutralisation with 4N sodium hydroxide, could be estimated in total, by the Somogyi method. If desired the fructosan content of the oligosaccharide fraction could be obtained by estimating the fructose in an aliquot of the hydrolysate, using Roe's method (41).

Fructosan.—The residue after ethanol extraction was shaken, overnight twice, with 500 ml. volumes of water at room temperature. The residue was well washed with water, then acetone, and dried overnight in an oven at 80°C before weighing. The water washings and extracts were combined and after suitable dilution the fructosan content determined by Roe's method.

Hemicelluloses, Cellulose and Lignin.—The residue after ethanol and cold water extraction was hydrolysed with N sulphuric acid (30 ml./g.) for one hour on a boiling waterbath. The residue was filtered off, washed well with water, then acetone and dried in an oven overnight at 80°C before weighing. The water washings and filtrate were made up to a specific volume (ca. 200 ml.) and an aliquot equivalent to 0·3 g. of material taken. This was made N with respect to sulphuric acid, and boiled under reflux for four hours. After cooling, ribose (ca. 20 mg.) as reference sugar was added, and neutralisation was effected by the addition of barium carbonate. The precipitate was filtered off, and the filtrate was evaporated to small volume. Two sets of chromatogram papers were run, one on solvent I, the other in solvent II. From these the weights of galactose, glucose, arabinose, and xylose present could be determined.

Using the residue obtained after N sulphuric acid hydrolysis, the ash content (on 100 mg.), and the protein content (on 50 mg.) (Kjeldahl), were determined. To the remainder, 72 per cent sulphuric acid (15 ml./g.) was added and hydrolysis allowed to proceed at room temperature for four hours. This was then diluted with water (23·5 ml. water per ml. 72 per cent acid used) and the solution boiled for three hours; after which the lignin was filtered off into an unweighed Gooch crucible, dried at 100°C overnight, then weighed before and after ignition. An aliquot of the 72 per cent hydrolysate equivalent to 100 mg. of the residue was taken. Ribose (ca. 25 mg.) was added as reference sugar, the solution was neutralised with barium carbonate, filtered, and the filtrate reduced in volume. Chromatogram papers were run in solvent I and glucose and xylose estimated.

The percentage of galactan, araban, xylan, cellulose and lignin in the original grass could be calculated as follows :—

Symbols :—

Except where otherwise stated weights are in grams.

1st res., *i.e.* residue after ethanol and water extraction.

2nd res., *i.e.* residue after ethanol, water and N sulphuric acid extraction.

G—dry weight grass sample.	
w—weight 1st res.	W—ditto 2nd res.
f—fraction of hydrolysate of 1st res. taken.	F—ditto 2nd res.
r—weight ribose added to 1st res. hydrolysate.	R—ditto 2nd res.
m—weight (mg.) monosaccharide *on paper* from w.	M—ditto 2nd res. (mg.).
x—weight (mg.) standard ribose *on paper* from w.	X—ditto 2nd res. (mg.).
A—% ash in 2nd res.	
N—% protein in 2nd res.	
H—weight of 2nd res. hydrolysed.	L—weight of ash free lignin.

Calculation :—

S—weight sugar from 72% hydrolysis $= \frac{MR}{XF} \times \frac{162^*}{180}$.

$$\text{P—\% recovery from 72\% hydrolysis} = \frac{(S_{glu} + S_{xyl})100}{\left[\frac{H(100 - N - A)}{100}\right]} - L$$

where $\left[\frac{H(100 - N - A)}{100}\right]$ is the ash and protein free weight of H.

Then the % *each polysaccharide* in dry grass is :—

$$\left[\left(\frac{mr}{xf} \times \frac{162^*}{180}\right) + \frac{100SW}{PH}\right] \times \frac{100}{G} \times \frac{\text{wt. water extracted grass}}{\text{wt. water extracted grass taken for N sulphuric acid hydrolysis.}}$$

Also % *lignin* in dry grass is :—

$$\frac{100LW}{HG} \times \frac{\text{wt. water extracted grass}}{\text{wt. water extracted grass taken for N sulphuric acid hydrolysis.}}$$

$^*\left(\frac{132}{150}\right)$ for pentosans.

SILAGE

The analytical methods employed for the extraction and determination of free sugars, fructosan and polysaccharide content of silage were similar to those described for grass analysis with only one modification. However, it was necessary in the case of silage to commence the analysis with a 50 g. sample (fresh weight).

The modification is as follows and the need for it arises since the free xylose present in silage is extracted during the 80 per cent ethanol treatment.

Two aliquots of this extract were taken and standard xylose added to one of them. They were both clarified, deionised, etc., in the usual way, and a set of chromatogram papers run in solvent I. The ratio of natural xylose to glucose was estimated from the papers to which no xylose had been added. The sugars were estimated as usual from the other set of papers. The weight of xylose on the paper equivalent to the amount of reference xylose added could then be calculated, and hence the weights of the sugars present in the extract, as previously.

2. *Nitrogenous constituents*

Analysis of Effluent

1. Total nitrogen content (T.N.). A 1 ml. aliquot of effluent was measured into a micro-Kjeldahl flask and 2 ml. N-free grade conc. sulphuric acid added along with 0·5 g. of catalyst (80 g. anhydrous potassium sulphate + 20 g. copper sulphate + 1 g. N-free sodium selenate). The digest was heated until clear and was then boiled vigorously overnight. After cooling, the digest was washed into the distillation apparatus, and made alkaline with 10 ml. of 10N potassium hydroxide. The ammonia was steam-distilled into excess N/70 hydrochloric acid. About 25-30 ml. of distillate was collected and then the back titration was carried out with N/70 sodium hydroxide.

2. Volatile nitrogen content (V.N.). A 2 ml. aliquot of effluent was measured into the distillation apparatus, and 3 ml. of 10·5 pH buffer (5 per cent borax in 0·5N sodium hydroxide) were added. The ammonia was steam-distilled into N/70 hydrochloric acid, 4 min. being allowed for each distillation. N/70 sodium hydroxide was again used in the back titration.

3. Total amide-nitrogen content ($CONH_2$-N). 2 ml. 2N H_2SO_4 was added to a 2 ml. aliquot of effluent contained in a boiling tube fitted with a stopper and capillary tube to act as a crude condenser. The digest was heated for 3 hours in boiling water, and the ammonia content determined as described above. Any ammonia found in excess of the V.N. value, after such treatment, will have been released from the amide groups of asparagine and glutamine.

4. Glutamine content. Glutamine hydrolyses completely to ammonia and pyrollidone-carboxylic acid when heated at pH 6·5 and 100°C for 2 hours.

The buffer used to make the necessary pH adjustment was composed of 1 part 0·2M potassium dihydrogen phosphate + 3 parts 0·10M borax. When mixed with an equal volume of effluent of pH 4·0-4·2 this buffer gave a resultant pH of 6·5.

In measuring glutamine, 5 ml. effluent was mixed with 5 ml. buffer and then heated in a boiling tube at 100°C for 2 hours. Free ammonia was then measured as above. Distillation time 4 mins.

5. Asparagine amide-nitrogen is the difference between total and glutamine amide-nitrogen.

Analyses of Fresh Grass and Silage

Three samples were always analysed for N distribution in the fresh undried material, since drying caused considerable proteolysis in fresh grass, and loss of volatile base in silages.

1. Total N was determined by adding about 300 ml. of conc. N-free sulphuric acid to 20 g. fresh grass or silage. The mixture was boiled gently in an iso-mantle until an homogeneous black liquid was obtained. This was cooled and made to volume with more conc. N-free sulphuric acid and a small aliquot then digested completely with the aid of the catalyst and the N determined as described under effluent T.N.

2. The soluble non-protein fraction of the grass or silage was obtained by extracting the material with boiling water. About 1 litre of boiling water was added to 100 g. material which was then squeezed or macerated for a few minutes and the supernatant liquid poured off through muslin, this was repeated twice and the combined filtrates concentrated in vacuo to the required volume. The solution was finally filtered through paper before analysis.

The various analyses on the soluble N fractions were then carried out as described under effluent.

3. *Dry matter*

SILAGE

A weighed quantity (about 100 g.) of chopped silage was added to a distillation flask and placed in a specially constructed electric oven at 100°C. A gentle current of air, preheated to 100°C, was passed through the silage and the water and volatile compounds were condensed in a Liebig condenser and collected in a weighed flask at − 10°C. Attached to the receiving flask were a weighed silica gel tube and a wash bottle of standard sulphuric acid.

The distillate was made up to a known volume and was analysed for volatile fatty acids, lactic acid and ammonia. The latter was determined using a micro-Kjeldahl steam distillation apparatus. The analysis of the organic acids was carried out according to the method described in a later section.

From a knowledge of the volatile constituents in the distillate the ‘ true ’ dry matter value of the silage could be calculated.

Effluents

The dry matter of the effluent was determined by correcting the apparent dry matter value, obtained by drying a 50 ml. sample overnight in an electric oven maintained at 100°C, for volatile acids and volatile nitrogen (calculated as NH_3). The procedure was as follows :—

(1) *Nitrogen in fresh effluent.*—As described earlier.

(2) *Nitrogen in dried effluent.*—10 ml. effluent were pipetted into a Kjeldahl flask and dried at 100°C in an electric oven overnight. A nitrogen determination was carried out on the residue. The difference between (1) and (2) was calculated as NH_3.

(3) *Apparent dry matter and ash.*—50 ml. effluent were dried in a silica basin at 100°C overnight. The residue was weighed, ignited and the ash weighed.

(4) *Volatile acids in fresh.*—To 50 ml. of effluent were added 10 ml. N/10 sulphuric acid and 140 ml. CO_2-free distilled water. The volatile acids were determined by the Weigner distillation technique as described by Smith and Comrie (42).

(5) *Volatile acids in dried effluent.*—50 ml. of effluent were pipetted into an aluminium foil basin moulded and supported in an evaporating basin. This was dried overnight at 100°C under similar conditions to the 'apparent dry matter determination.' The aluminium foil with dried residue was transferred to a 750 ml. flask and after adding 190 ml. CO_2-free water and 10 ml. N/10 sulphuric acid, a volatile acid determination was carried out by the Weigner method.

True dry matter = Apparent dry matter + volatile NH_3 + acids lost on drying.

4. *Acids*

Volatile fatty acids in fresh silage

The volatile fatty acids in the fresh silage were determined by the method of Wiseman and Irvin (11).

A sulphuric acid extract of the silage was prepared by allowing 50 g. of fresh silage to stand in contact with 50 ml. of 0·6N sulphuric acid for a week in a stoppered bottle under refrigeration at 0°C. The extract was then centrifuged and a 2 ml. sample passed through a Celite column using alphamine red-R as internal indicator. The acids were eluted from the column with mixtures of petroleum ether (b.p. 63-70°C) and acetone in varying proportions. After elution the acids were titrated with $\frac{N}{200}$ sodium hydroxide using cresol red as indicator.

Lactic acid in fresh silage

The lactic acid content in the silages was determined by the method of Elsden and Gibson (12) and was as follows :—

An aqueous extract of the silage was prepared by macerating 50 g. of silage with 250 g. of water for 3 minutes. After filtering the extract through muslin an aliquot was purified by treatment with copper sulphate and calcium hydroxide and deproteinised by tungstic acid precipitation.

Aliquots were then oxidised with ceric sulphate in an Elsden and Gibson apparatus and the resultant acetaldehyde trapped in excess sodium bisulphite; after removal of the excess sodium bisulphite the acetaldehyde bisulphite compound was decomposed with sodium bicarbonate and the liberated bisulphite titrated against standard solution in the usual way.

Bacteriological Methods

Aseptic precautions were maintained throughout all the manipulations so as to avoid the introduction of extraneous micro-organisms. Such precautions are deemed especially necessary when small samples of herbage are being fermented as in the case of the laboratory-made silages, since under favourable conditions the growth of any one type of organism may influence the course of the fermentation even though the number of that organism present initially is negligible. Sheep shears, which can readily be sterilised in the hot air oven, proved useful for cutting herbage and silage samples. The macerator and jars which were used to disintegrate the grass and silage were washed thoroughly after use and sterilised by rinsing with ethanol and flaming. The rubber pad surrounding the spindle of the macerator was difficult to sterilise, therefore it was covered before each maceration with a sterile disk of thin rubber sheeting, so slotted at the centre that it fitted closely round the spindle. Sterility tests, which were carried out regularly by running the macerator with 300 ml. of sterile water and then plating 1 ml. of the water on each of several media, rarely yielded more than an occasional bacterial colony when these precautions were taken.

Preparation of silage for examination.—All the material of a laboratory silage was withdrawn from the tube by means of a sterile metal corkscrew which had an elongated shank sufficiently long to permit the end to reach almost to the foot of the tube. Cutting the silage into smaller pieces at this stage facilitated maceration.

Media and methods.—Serial decimal dilutions of the macerate were made in sterile water and were used for the inoculation of five media which yield counts of different bacterial groups.

Medium 1.—*Glucose yeast agar*; peptone (Evans), 1 g.; meat extract (Lemco), 1 g.; yeast autolysate, 5 ml.; glucose, 0·5 g.; agar (Davis), 1·5 g.; tap water, 100 ml.; pH 6; sterilised momentarily at 22 lb./sq. in. To prepare the yeast autolysate, 1 kg. brewer's yeast in 1 l. of water was held at 50°C for 24 hr.; after centrifugation, the supernatant fluid was sterilised in bottles momentarily at 22 lb./sq. in.

Poured plates were incubated in air for 5 days at 30°C. The medium might be expected to support the growth of most of the silage bacteria other than the obligate anaerobes.

Medium 2.—*Acetate agar* (Keddie, 43): peptone (Evans), 1 g.; meat extract (Lemco), 1 g.; yeast autolysate (as in medium 1), 5 ml.; tomato extract, 20 ml.; glucose, 1 g.; Tween 80, 0·05 ml.; agar (Davis), 1·5 g.; tap water, 100 ml. The peptone, meat extract and agar were dissolved together, the yeast autolysate and tomato juice then added and the pH value adjusted to 5·4; after filtration the glucose and Tween 80 were added and the medium was bottled and sterilised at 15 lb./sq. in. for 15 min. To prepare the tomato extract, 1 kg. tomatoes and 450 ml. of water were steamed for 30 min.; the pulp was then squeezed through muslin.

Just immediately before pouring plates, 10 ml. of 2M acetic acid/sodium acetate buffer of pH 5·4 were added to 90 ml. of the molten medium. The final pH should be 5·4 $\pm$ 0·05. After the inoculated agar had solidified a second layer of acetate agar was added. Plates were incubated in air for 5 days at 30°C. This medium was used for counting lactobacilli. It was essential to identify colonies by isolation and microscopical examination of the isolates, since *Leuconostoc* and *Pediococcus* can grow on the medium.

Medium 3.—*Lactate agar* ; ammonium lactate syrup (c. 61 per cent), 1 ml.; glucose, 0·2 g.; L-glutamic acid, 0·2 g.; potassium phosphate (K_2HPO_4), 0·1 g.; magnesium sulphate, 0·02 g.; washed shred agar, 1·5 g.; tap water, 100 ml.; pH 6; sterilised at 22 lb./sq. in. Just before use crystal violet was added to make a final concentration of 1/500,000 (1 ml. of sterile 1/5000 solution added to 100 ml. of molten medium) in order to inhibit those Gram-positive bacteria which might develop on the medium. Since differentiation by colony type was not possible in poured plates, 0·1 ml. of dilutions of the macerate was spread over the surface of dried plates of the medium which were then incubated for 3 to 5 days for a count of Gram-negative bacteria.

Medium 4.—*Medium for lactate-fermenting anaerobes* (Rosenberger 44): sodium lactate syrup (c. 70 per cent), 1·5 ml.; sodium acetate, hydrated, 0·8 g.; ammonium sulphate, 0·05 g.; yeast autolysate, 3 ml.; biotin, 0·01 μg.; p-aminobenzoic acid, 10 μg.; L-cysteine hydrochloride, 0·05 g.; thioglycollic acid, 0·05 ml.; a mineral supplement; resazurin, 0·5 mg.; agar, 0·2 g.; tap water, 100 ml.; pH 6; sterilised at 22 lb./sq. in. The agar was dissolved before adding the other ingredients.

Medium 5.—*Medium for proteolytic anaerobes* (Rosenberger, 44): peptone (Evans), 1·5 g.; gelatin, 12 g.; yeast autolysate, 1 ml.; L-cysteine hydrochloride, 0·05 g.; resazurin, 0·5 mg.; tap water, 100 ml.; pH 7; sterilised at 22 lb./sq. in. Before inoculating media (4) and (5) the tubes were placed in boiling water for 10 min. and then cooled to 45°C. The inoculum was placed deep in the medium; mixing and introduction of air was avoided. After inoculation the tubes were chilled to solidify the agar or gelatin and the medium was covered to a depth of 3 cm. with a seal of: agar, 1 g.; L-cysteine hydrochloride, 0·05 g.; thioglycollic acid, 0·05 ml.; resazurin, 0·5 mg.; water, 100 ml.; pH 7; sterilised at 22 lb./sq. in. The cultures were incubated in air for 7 days at 30°C and examined as follows: (*a*) Lactate fermentation in medium (4) is recognised by gas formation sufficient to raise the seal at least 2 cm. and by a rise in pH which is detected by testing some of the culture with bromo-thymol-blue on a spot plate. (*b*) Protein breakdown in medium (5) is shown by liquefaction of gelatin and by strong reactions for ammonia and hydrogen sulphide in spot tests with Nessler's reagent and with lead acetate. A vanillin test (Roessler and McClung, 45) assists the recognition of *Clostridium sporogenes*. Some but not all of these reactions may be produced by non-proteolytic clostridia and by other bacteria (Rosenberger, 46). Dilutions of the macerate were inoculated into media (4) and (5) in triplicate, and the most probable numbers estimated using the tables of Hoskins (47).

Other media which were found useful in the further characterisation of the silage bacteria are noted below :—

Tween semi-solid agar ; peptone (Evans), 1 g. ; meat extract (Lemco), 1 g. ; yeast autolysate, 5 ml. ; tomato extract, 20 ml. ; glucose, 1 g. ; Tween 80, 0·05 ml. ; agar (Davis), 0·1 g. ; tap water, 100 ml. The peptone, meat extract and agar were dissolved ; the yeast autolysate and tomato extract then added and the pH adjusted to 6 ; after filtration the glucose and Tween 80 were added and the medium was distributed in 4·5 ml. amounts in small tubes and sterilised at 22 lb./sq. in. This medium was used for the isolation of organisms from acetate agar and from glucose yeast agar.

Basal medium for fermentation reactions of some lactobacilli : peptone (Evans), 0·5 g. ; meat extract (Lemco), 0·5 g. ; yeast autolysate, 5 ml. ; Tween 80, 0·05 ml. ; tap water, 100 ml. ; pH 6·8. The basal medium was used for the growth of lactobacilli prior to fermentation tests made in the same medium with the addition of filter-sterilised sugars added aseptically to make a concentration of 1 per cent. Bromo-cresol purple was used as indicator.

Glucose agar : peptone (Evans), 0·5 g. ; meat extract (Lemco), 0·5 g. ; agar, 1·5 g. ; tap water, 100 ml. ; glucose, 1 g. ; pH 6·8. Bromo-cresol purple was added as indicator and the medium was tubed in 10 ml. amounts and sterilised at 22 lb./sq. in. The growth of Gram-negative organisms on this medium permits a preliminary grouping on the basis of oxygen requirement and action on glucose.

IDENTIFICATION OF BACTERIA

In addition to counting the main groups of bacteria which developed in the silages, colonies from the plates of highest dilution giving suitable counts were picked for further identification. Growths from glucose yeast agar and from acetate agar were transferred to Tween semi-solid agar and were examined after incubation at 30°C for 2 days. Nigrosin films from these cultures gave good morphological differentiation of streptococci, lactobacilli and pediococci. Films made from growths on solid media do not always show this distinction satisfactorily. At the same time in this medium the hot wire test provided a preliminary division of the cultures into homofermentative and heterofermentative types, while the appearance of the undisturbed growth gave some indication of the oxygen tolerance of the organism. Pure cultures for further study were obtained by replating on glucose yeast agar, the same medium being suitable for demonstrating different colony types among the species of lactobacilli.

Growths from lactate agar were first inoculated into deep tubes of glucose agar containing bromo-cresol purple indicator. Incubation at 30°C for three days yielded information which provided a preliminary separation of aerobes and facultative anaerobes. A further division could be made into groups according to the action on glucose, *i.e.* production of acid alone, or acid and gas with or without reversion of the colour of the indicator at the top of the medium. Organisms producing coloured or fluorescent growths could be distinguished readily.

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DETERMINATION OF DRY MATTER AND VOLATILES IN SILAGE*

By P. McDONALD and W. A. DEWAR

An apparatus designed to collect the volatile constituents produced during the drying of foods has been used to investigate the losses of volatiles from silages dried at 100°. In 28 silages examined the mean volatility of acetic acid was 87·9% and of butyric acid 89·4%. In all samples, lactic acid was found to be volatile, the percentage volatility ranging from 1·4 to 16·4. Appreciable losses of nitrogen occurred during the drying of silages with high pH values.

Introduction

The determination of dry matter in a feeding stuff is usually carried out by heating a sample overnight in an oven at a temperature about 100°, the loss of weight being considered to be water and the residue to be dry matter. This method cannot be applied to the accurate determination of dry matter in foods containing volatile constituents other than water. Silage comes into this latter category and a number of workers, including Watson & Ferguson[1] and Woodman[2] have drawn attention to the importance of taking into consideration the volatile compounds lost on heating, in order to assess the true losses of dry matter occurring during the ensilage process. Watson[3] has reviewed the nature of these volatile compounds, which include acids, bases and alcohols.

A number of suggestions for determining the 'true' dry matter value of silage have been proposed and one of the most widely accepted methods is to correct the 'apparent' dry matter for volatile fatty acids and nitrogen (calculated as NH_3) lost on drying. The volatile loss is derived from an analysis of the fresh and dried silages.[1, 4, 5] Perkins[6] has described an alternative method based on the Dean & Stark toluene-distillation technique in which the water is

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collected and measured volumetrically. The former method does not take into account the volatility of lactic acid and in the distillation technique the moisture content will be over-estimated unless the aqueous distillate is analysed for volatile constituents.

The purpose of the present investigation was to study the nature of the volatiles lost during the oven-drying of silages by collecting and analysing the distillates produced.

Experimental

Apparatus

The apparatus used for drying the silages and collecting the distillates is shown in Fig. 1 and consists of a round-bottomed, two-necked 1000-ml. flask (A) housed in a thermostatically controlled electric oven. The main outlet from the flask is connected through an insulated stillhead (B) passing through an opening in the oven roof to a Liebig condenser (C) and thence through a long delivery tube (D) into a 300-ml. Kjeldahl flask (F). During a determination the Kjeldahl flask is enclosed in a wide-mouthed vacuum flask containing a mixture of ice and salt at $-10°$. A gentle current of pre-heated, CO_2-free and moisture-free air is pumped through the side-neck (J) into the two-necked flask containing a weighed amount of chopped silage. The air leaves the apparatus through pre-weighed absorption tubes containing silica gel (G) and soda lime (H). Finally the air is passed through a gas wash bottle containing standard H_2SO_4.

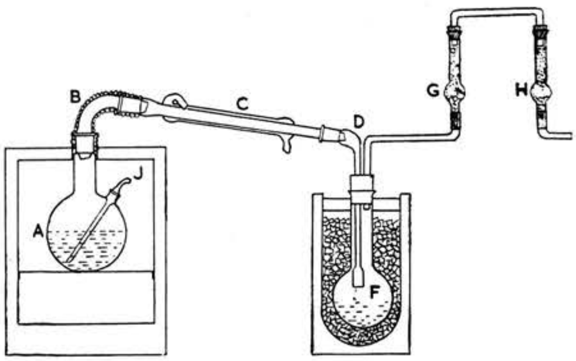

FIG. 1.—*Apparatus used for collection of volatile matter*

Analysis of distillate

Volatile fatty acids (formic, acetic, propionic and butyric) were determined by the column chromatographic method of Wiseman & Irvin;[7] lactic acid by the ceric sulphate oxidation method of Elsden & Gibson;[8] total nitrogen and ammonia by the micro-Kjeldahl method and alcohol by Winnick's microdiffusion technique.[9]

Before investigations of silage, recoveries of volatile fatty acids, lactic acid and ammonia were determined in the apparatus described above. During these determinations the oven temperature was maintained at 100° and the rate of air flow kept constant at 0·5 cu. ft./h. In the determination of volatiles in silage a similar drying temperature and air flow were maintained. For this determination about 100 g. of chopped silage were accurately weighed into the distillation flask. On completion of the drying process the silage residue, distillation and absorption tubes were weighed and the distillate was analysed for formic, acetic, propionic, butyric and lactic acids, nitrogen and alcohol.

Results

Recoveries of pure solutions are shown in Table I, and it is clear that within the limits of experimental error, all volatiles were recovered during distillation.

Twenty-eight samples of grass silages, made without additives, ranging in pH value from 3·7 to 5·2 were examined. In all cases the recovery of total volatiles in the receiver and silica gel tube accounted for the losses from the distillation flask. The percentage recoveries (by weight)

Table I

Recoveries of pure solutions in distillates

	Individual acids or ammonium hydroxide, %		Mixture of acids and ammonium hydroxide %	
Acetic acid	99·3	98·8	98·8	99·6
Propionic acid	99·1	99·8	99·2	99·7
Butyric acid	99·3	98·5	98·7	99·4
Lactic acid	67·0[a]	67·7[b]	69·0[c]	70·2[d]
Ammonium hydroxide	95·3	94·8	94·7	93·7

Residual lactic acid after hydrolysis in distillation flask: [a] 32·7%, [b] 32·7% [c] 30·4% [d] 28·3%

ranged from 99·60 to 100·36 with a mean recovery of 99·94% for the 28 silages examined. The CO_2 absorption tube did not increase in weight and ammonia was not detected in the acid wash bottle in any of the determinations. The results of the analysis of fresh silages and distillates are given in Table II. In addition to the 'apparent' dry matter values, i.e., the residues after drying for 18 h. at 100° in the distillation apparatus, the corrected dry matter values are given and these were obtained by including the total volatiles as determined in the distillates.

Table II

Composition of silages

No.	pH	mg./100 g. of fresh silage								%		
		Nitrogen		Acetic acid		Butyric acid		Lactic acid		Dry matter		$\frac{b-a}{b} \times 100$
		Total	Volatile	Total	Volatile	Total	Volatile	Total	Volatile	Apparent *a*	Corrected *b*	
1	3·7	586	0	343	331	0	0	1827	156	18·60	19·09	2·55
2	3·7	336	7	373	338	32	30	2004	215	16·32	16·91	3·51
3	3·7	478	9	356	342	8	8	2315	191	20·51	21·07	2·66
4	3·8	568	0	345	316	0	0	1660	148	19·29	19·75	2·35
5	3·8	589	0	358	313	0	0	1579	168	18·33	18·81	2·56
6	3·8	346	0	367	348	80	70	1446	93	16·14	16·67	3·18
7	3·8	326	39	259	217	135	123	1717	104	19·58	20·22*	3·17
8	3·9	571	0	345	333	0	0	1661	146	18·80	19·28	2.48
9	3·9	326	6	368	298	213	206	1418	91	14·70	15·30	3·92
10	3·9	379	5	253	218	24	20	1821	128	15·12	15·49	2·40
11	3·9	374	2	234	186	29	23	1744	168	16·42	16·80	2·25
12	4·0	364	2	264	194	29	26	1611	157	61·98	17·34	2·08
13	4·1	297	8	307	284	336	330	825	54	14·07	14·75	4·61
14	4·1	366	7	266	235	30	23	1736	108	15·97	16·34	2·26
15	4·1	345	12	261	238	23	24	2097	98	18·62	19·07	2·36
16	4·3	669	95	298	257	0	0	1989	250	18·22	18·84	3·30
17	4·3	373	44	277	257	67	60	2106	313	35·42	36·13†	1·98
18	4·3	292	7	473	368	27	22	684	112	23·08	23·81**	3·09
19	4·35	647	35	296	275	0	0	1808	216	18·22	18·75	2·84
20	4·5	668	61	397	357	0	0	1914	247	16·93	17·61	3·85
21	4·7	473	31	388	362	380	312	222	3	19·91	20·62	3·44
22	4·7	477	92	315	229	429	424	198	27	23·46	24·35	3·66
23	4·8	229	21	210	168	269	253	1619	11	14·33	14·79	3·11
24	4·9	645	152	319	284	0	0	1615	265	17·57	18·30	3·99
25	4·9	427	12	747	589	96	55	2360	47	17·44	18·14	3·86
26	4·9	253	51	661	551	370	299	20	1	15·54	16·45	5·55
27	5·1	362	133	344	333	661	668	265	11	16·73	17·90	6·56
28	5·2	254	123	555	546	698	629	105	8	13·80	15·13	8·80

* Including 0·15% volatile propionic acid
† Including 0·04% volatile propionic acid
** Including 0·23% volatile propionic acid

Discussion

The volatile N expressed as % of the total N ranged from 0 to 48·4% in the 28 samples examined. A number of workers (e.g., [1, 10, 11]) have commented on the direct relationship between volatile N and pH value and these results tend to confirm this finding. This relationship appears to be better at high pH levels and Smith & Comrie[11] stated that the critical zone occurs about pH 4·5. Unfortunately in the present studies only eight silages had pH values above this level and only two of these were above pH 5·0. Schoch[4] has shown that losses of volatile nitrogen can be considerable above pH 5·0. At high pH levels there appears to be an inverse relationship between volatile N and lactic acid content, a relationship which would be expected from a knowledge of the fermentation reactions occurring during ensilage. Silages with pH below 4·0 have negligible volatile N contents, with the exception of sample 7. This silage was unusual in containing propionic acid as well as an appreciable amount of butyric acid.

Acetic acid was present in all the silages examined. The volatility of this acid, during the drying process, ranged from 72·7 to 98·4% with a mean value of 87·9% for the 28 silages examined. Watson[3] has studied the volatility of fatty acids in silages and in an examination of 67 silages found that the total volatile acids (calculated as acetic acid) ranged from 50·1 to 90·2% of the total amount present, with a mean value of about 77%. In these experiments, however, the silages were dried at 98° which is slightly lower than the temperature used in the present studies. The total amounts of acetic acid present in silage can be considerable; in this investigation, 18 silages had total acetic acid values above 0·3% of the fresh material. It is clear from the results that this acid is the main volatile component of most silages.

Butyric acid is usually absent or present in only small amounts in well-preserved silages. In the 20 samples examined which had pH values below 4·5, 13 contained butyric acid although in only three cases did the amounts present exceed 0·1% of the fresh silages and two of these samples were associated with exceptionally wet material. The percentage volatility of this acid in the silages ranged from 57·3 to 104 with a mean value of 89·4%. In the two samples of pH value above 5·0, the quantity of volatile butyric acid present was higher than that of any other volatile constituent. This association of high butyric acid content with high pH is well known and is a reflection of the type of bacterial fermentation which has occurred.

Propionic acid was detected in only three silages, in samples 7, 17 and 18. The percentage volatilities of this acid in these samples were 118, 92 and 86 respectively. In view of the unreliability of one of these values and because of the few samples involved, it is impossible to comment on these results.

Small quantities of formic acid have been detected in silages (Langston *et al.*[12]) but this acid was not found in any of the silages examined here.

Lactic acid is usually regarded as being non-volatile but it was recovered in the distillate from all silages. The volatility of the acid was also demonstrated in the preliminary experiments with pure solutions. Smith[13] and Woodman[2] have already drawn attention to the importance of considering this property in silage studies. The percentage volatility of lactic acid in the 28 silages ranged from 1·4 to 16·4 with a mean value of 8·7%. These values are lower than those found for acetic and butyric acids, but because lactic acid is usually present in larger quantities than the fatty acids in well-preserved silages, the losses during drying can be significant. The reason why lactic acid is frequently ignored in dry matter corrections may be due to the difficulties in assessing the volatility of the acid since difference calculations based on analysis of fresh and dried silages are not valid owing to the formation of lactic anhydride and lactide during heating.

Alcohols have been isolated from silage by many workers but not exceeding about 0·5% of the fresh silage (Barnett[14]). Alcohols, as determined by Winnick's micro-diffusion method, were not detected in any of the silages examined.

From a comparison of the apparent dry matter values with the corrected dry matter figures given in Table II, it is clear that the losses of volatiles during drying are of importance in assessing the true dry matter of silages. These differences become more significant in balance experiments where silage losses are calculated. The total volatiles ranged from 1·98 to 8·80% of corrected dry matter and were highest in the silages with high pH containing relatively large amounts of butyric acid and volatile N.

Conclusions

It is clear from an analysis of the distillates produced during the drying of silages that considerable losses of volatiles occur and these must be considered in order to arrive at a true value for dry matter. The high losses of acetic and butyric acids which occur during the drying of silages have been stressed by a number of workers, although the volatile nature of lactic acid under normal drying conditions is frequently overlooked. The apparatus used in this experiment for the collection of silage distillates can be used for the determination of dry matter although the necessity for analysing the distillate makes the method tedious for routine purposes.

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4

DETERMINATION OF DRY MATTER IN SILAGE BY DISTILLATION WITH TOLUENE

By W. A. DEWAR and P. McDONALD

The determination of dry matter in silages by distillation with toluene gives satisfactory results when an allowance is made for volatiles present in the aqueous distillate. A simple correction procedure, involving a single titration of the distillate, has been developed, and the method is considered suitable for routine purposes.

Introduction

In an earlier paper,[1] an apparatus was described which enabled the volatile constituents, produced during the oven-drying of silage, to be collected and determined quantitatively. An examination of 28 silages, ranging in pH value from 3·7 to 5·2, showed that appreciable losses of lower fatty acids, lactic acid and ammonia occurred during oven drying. The apparatus used in these studies could be used for the accurate determination of dry matter in silage, but for routine purposes the method was considered tedious, since it required a detailed analysis of the distillate for volatile components. Since there is a need for a rapid and accurate method of determining dry matter in silage, an alternative method was sought and the present paper summarises the results from a study of the toluene distillation technique which has already been suggested as a possible alternative to the normal oven-drying method.[2–4]

The apparatus used in the toluene distillation technique has been described by Bidwell & Sterling.[5] The sample under test is heated in a distilling flask with an excess of toluene. The distillate containing water and toluene is condensed and collected in a graduated receiver or trap, so designed that the excess toluene flows back into the boiling flask. Distillation is continued until all the water has been driven off from the sample and the volume of water in the trap is then measured.

The accuracy of the method depends largely upon the precision with which the volume of water may be measured in relation to the sample size. With very wet materials, such as fresh herbage or silage, the accuracy in measuring the volume of aqueous distillate can be increased by using a trap provided with an ovoid-shaped bulb at its lower end and graduated stem above, similar to that described by Evans & Fetzer.[6] This type of trap allows a larger sample size to be used.

Where the toluene method has been used previously in silage studies, the volatiles present in the distillate have either been ignored or considered to be of little importance; but it is desirable to know what the possible errors are. The purpose of the present investigation was to study the magnitude of these errors and to decide if the toluene distillation technique would be suitable as an accurate routine method for determining dry matter in silage.

Experimental

Apparatus

The distillation apparatus used in these studies is shown in Fig. 1. The trap was made by cutting the stem from a conventional Bidwell & Sterling trap[5] of capacity 8 ml. and fusing it to an ovoid-shaped bulb of about 50 ml. capacity.

The trap was recalibrated and was found to be suitable for measuring volumes of water between 51·70 and 58·20 ml. to the nearest 0·05 ml. It was connected by a reduction adapter, with ground joints, to a 1000-ml. wide-mouthed, round-bottomed flask which was supported in an Electro-thermal heating mantle fitted with a sensitive temperature control. A single-surface water condenser stoppered with a silica gel absorption tube, to exclude atmospheric moisture, was attached to the top of the trap.

Recovery test with volatiles

Known volumes of water and aqueous solutions of acetic, propionic, butyric and lactic acids, ammonia and ethanol were separately distilled in the apparatus, with about 400 ml. of toluene, until the volume of aqueous distillate collected in the trap was constant. Details of the procedure are given below.

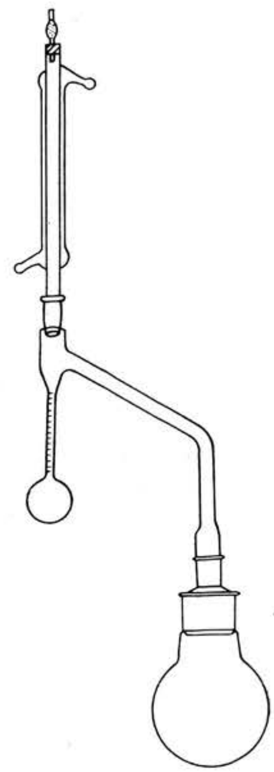

FIG. 1.—*Toluene distillation apparatus*

Volumes occupied by volatile materials in aqueous solution

Before applying the distillation technique to the determination of dry matter in silage, it was necessary to know the volume occupied by individual volatiles in aqueous solution. These volumes were found by determining the densities at 20° of known concentrations of aqueous solutions of the volatile substances mentioned in the previous paragraph. Since the density of water at 20° is 0·9982 g./ml., the volume of water, and hence the volume occupied by a known weight of solute, could be calculated.

The volume occupied by ammonia in 1% v/v acetic acid was determined by a different method in which the contraction in volume was measured in a graduated flask after the addition of ammonia solution to dilute acetic acid.

Application of the toluene distillation method to silage

Determinations of dry matter were carried out on 16 different silage samples ranging in pH value from 3·7 to 5·4. These silages had all been made in farm silos from grass or grass/clover mixtures without additives. The procedure was as follows:

Samples of 65 g. of chopped silage were accurately weighed into the distillation flask and heated with about 400 ml. of toluene in the apparatus. The distillation was continued for 7–8 h. at a rate of 2–3 drops per second. At the end of this period, a fine jet of toluene was directed down the condenser to remove the last traces of water and the distillation continued for a further 15 min.

Before the volume of water was read the graduated trap was immersed in a water bath at 20° for 20 min. The apparatus was disconnected and the aqueous layer was transferred into a 100-ml. graduated flask and made up to the mark with CO_2-free distilled water.

The total acid content of each distillate was determined on an aliquot of the diluted distillate by the method of Foreman,[7] which depends on the fact that in 80% alcoholic solution ammonia is neutral to phenolphthalein. Samples of 20 ml. of the diluted distillates were therefore diluted with 80 ml. of neutral ethanol and titrated with 0·1N-NaOH to the phenolphthalein end point.

In addition to the Foreman titration, samples of the distillate were analysed for individual volatile fatty acids, lactic acid, ammonia and alcohol by the methods described in an earlier paper.[1]

Oven-dry matter determinations

In order to compare the toluene distillation technique with the oven-drying method, the dry matter values of the 16 silages were determined in the apparatus as described in an earlier paper.[1] This method enables values for volatile matter, and hence for corrected dry matter, to be determined for the silages.

Results

Recoveries of water and aqueous solutions of lower fatty acids, lactic acid, ammonia and ethanol in the distillates after toluene distillation using the apparatus described earlier, are shown in Table I. Almost complete recoveries of water were obtained in all tests.

Table I

Recoveries of volatiles in aqueous distillates from toluene distillation of pure solutions

	Concentration, g./100 ml.			% recovered in distillate			Mean % recovered in distillate
	a	b	c	a	b	c	
Acetic acid	0·185	0·370	0·740	96·8	97·0	97·1	96·9
Propionic acid	0·137	0·274	0·548	65·3	65·9	65·0	65·4
Butyric acid	0·181	0·362	0·724	20·2	18·5	19·0	19·2
Lactic acid	0·301	0·903	1·505	6·5	7·9	7·7	7·1
Ammonia	0·038	0·076	0·114	91·4	90·8	90·0	90·5
Ethanol	0·117	0·234	0·351	85·4	82·1	90·0	85·8
Water	—	—	—	99·96	99·92	99·94	99·94
				99·96	99·92	99·94	

The volumes occupied by the various volatile compounds in aqueous solution at three concentration levels were studied, viz., at 0·2, 0·6 and 1·0 g. of solute per 100 ml. of solution. Within this range, good agreement was obtained for individual compounds. The volumes occupied by 1 g. of acetic, propionic and butyric acids were 0·864, 0·916 and 0·958 ml. respectively. The corresponding value for 1 g. of lactic acid was 0·778 ml. and for alcohol 1·219 ml. The value for ammonia in acetic acid solution was 0·247 ml.

The 16 silages were examined by both the oven distillation method and the toluene distillation technique. The composition of these silages and the volatiles produced during oven distillation are shown in Table II. Alcohol and propionic acid were absent from most of the silage samples.

The weights of volatiles in the aqueous distillates obtained from the 16 silages examined by toluene distillation are shown in Table III. In addition to the weights of volatiles, the volumes have also been calculated using the appropriate factors described earlier. The percentage volatilities of the various acids have been calculated and the mean values for the

16 silages were as follows: acetic, 87·7; propionic, 55·7; butyric, 7·3; and lactic acid, 3·6. These are lower than the corresponding figures obtained on pure solutions. The values in the last column of Table III (F) have been calculated in each case by dividing the total volume due to volatiles (acid + ammonia) in the distillate by the volume of 0·1N-NaOH, equivalent to the total acids present. The total acid titre can be determined by means of the Foreman titration described earlier. 'F' is therefore a factor used to calculate the volume of volatiles in the distillate from the titratable acidity. The mean value of F for the 16 silages was 0·00555.

Table II

Composition of silages and volatiles produced during oven distillation method

No.	pH	mg./100 g. of fresh silage								Dry matter, %	
		Nitrogen		Acetic acid		Butyric acid		Lactic acid			
		Total	Volatile	Total	Volatile	Total	Volatile	Total	Volatile	Apparent	Corrected
1	3·7	397	nil	298	291	nil	nil	1928	187	15·60	16·08
2	3·8	488	nil	261	255	161	152	1818	145	20·65	21·20
3	3·8	422	nil	303	285	nil	nil	2046	256	18·75	19·37[a]
4	3·8	294	10	302	281	20	15	1922	213	19·65	20·27[b]
5	3·9	378	nil	401	375	nil	nil	2021	130	20·81	21·32
6	4·0	330	10	341	320	nil	nil	1321	148	18·38	18·86
7	4·1	310	33	358	328	63	60	2020	199	18·57	19·28[c]
8	4·2	295	15	361	337	nil	nil	1311	65	16·78	17·20
9	4·5	455	36	505	490	317	300	1427	110	21·77	22·71
10	4·6	471	55	419	400	266	243	33	nil	13·81	14·51
11	4·7	405	98	390	364	nil	nil	605	55	21·75	22·27
12	4·9	268	73	491	460	335	315	299	20	15·62	16·49
13	5·0	555	91	789	741	610	576	96	10	13·61	15·08[d]
14	5·0	458	74	576	489	515	498	13	nil	12·80	13·86
15	5·2	635	120	434	389	615	602	19	nil	16·67	17·79
16	5·4	647	162	649	641	633	607	13	nil	15·82	17·43[e]

[a] including 0·08% volatile alcohol
[b] including 0·11% ,, ,,
[c] including 0·09% volatile propionic acid
[d] including 0·05% ,, ,, ,,
[e] including 0·20% ,, ,, ,,

Table III

Volatiles in aqueous distillates obtained by the toluene distillation method from 65-g. samples of fresh silage

No.	Ammonia		Acetic acid		Butyric acid		Lactic acid		Total volume of acids and ammonia in distillate, ml. (x)	ml. 0·1N-alkali equivalent to acids in distillate (y)	$\frac{x}{y}$(F)
	wt., g.	volume, ml.	wt., g.	volume, ml.	wt., g.	volume, ml.	wt., g.	volume, ml.			
1	nil	—	0·177	0·153	nil	—	0·038	0·029	0·182	33·7	0·00540
2	nil	—	0·153	0·132	0·010	0·010	0·038	0·030	0·172	30·8	0·00558
3	nil	—	0·179	0·155	nil	—	0·031	0·024	0·179	33·3	0·00538
4	0·005	0·001	0·171	0·148	nil	—	0·028	0·022	0·171	31·6	0·00541
5	nil	—	0·234	0·202	nil	—	0·034	0·026	0·228	42·7	0·00540
6	0·006	0·002	0·188	0·163	nil	—	0·040	0·031	0·194	35·7	0·00585
7[a]	0·018	0·004	0·204	0·176	0·003	0·003	0·027	0·021	0·239	42·3	0·00565
8	0·008	0·002	0·208	0·180	nil	—	0·029	0·023	0·205	37·9	0·00544
9	0·018	0·004	0·299	0·258	0·012	0·012	0·025	0·019	0·293	54·0	0·00543
10	0·031	0·008	0·251	0·217	0·013	0·013	nil	—	0·238	43·3	0·00550
11	0·054	0·013	0·227	0·196	nil	—	0·005	0·004	0·213	38·4	0·00555
12	0·040	0·010	0·279	0·241	0·012	0·012	0·004	0·003	0·266	48·3	0·00549
13[b]	0·051	0·013	0·435	0·376	0·027	0·026	0·004	0·003	0·435	78·6	0·00554
14	0·038	0·009	0·282	0·244	0·025	0·024	nil	—	0·277	49·8	0·00556
15	0·072	0·018	0·229	0·198	0·035	0·034	nil	—	0·250	42·2	0·00569
16[c]	0·104	0·025	0·345	0·317	0·028	0·027	nil	—	0·431	70·0	0·00615

[a] Propionic acid, total 0·038 g., volume 0·035 ml.
[b] Propionic acid, total 0·019 g., volume 0·017 ml.
[c] Propionic acid, total 0·069 g., volume 0·062 ml.

The values for dry matter determined by the oven distillation method and the toluene distillation technique are summarised in Table IV. The uncorrected dry matter values are

Table IV

Comparison of percentage dry matter values obtained in oven distillation method and in toluene distillation method

No.	Oven distillation apparatus at 100°			Toluene distillation procedure			
	Uncorrected (a)	Corrected (b)	$\frac{b-a}{b} \times 100$	Uncorrected (c)	$\frac{b-c}{b} \times 100$	Corrected using 'F' (d)	$\frac{b-d}{b} \times 100$
1	15·60	16·08	2·99	15·88	1·24	16·21	−0·81
2	20·65	21·20	2·59	20·84	1·70	21·07	0·61
3	18·75	19·37	3·20	18·92	2·32	19·20	0·87
4	19·65	20·27	3·06	19·93	1·68	20·20	0·35
5	20·81	21·32	2·39	21·00	1·50	21·37	−0·24
6	18·38	18·86	2·55	18·53	1·75	18·84	0·16
7	18·57	19·28	3·68	19·07	1·09	19·42	−0·73
8	16·78	17·20	2·44	16·92	1·63	17·23	−0·17
9	21·77	22·71	4·14	22·31	1·76	22·77	−0·26
10	13·81	14·51	4·82	14·16	2·25	14·51	0·00
11	21·75	22·27	2·33	21·84	1·93	22·16	0·49
12	15·62	16·49	5·28	15·84	3·94	16·27	1·34
13	13·61	15·08	9·75	14·47	4·04	15·13	−0·33
14	12·80	13·86	7·65	13·46	2·89	13·96	−0·72
15	16·67	17·79	6·30	17·31	2·70	17·81	−0·10
16	15·82	17·43	9·24	16·88	3·15	17·46	−0·20

the values obtained when the volatiles are not taken into account. The 'corrected' values for dry matter given under the 'toluene distillation' column in Table IV have been obtained by multiplying the total titre in ml. of 0·1N-alkali, obtained from a Foreman titration of the distillate, by the mean factor (F) 0·00555. This volume is subtracted from the observed volume of distillate to give the true volume of water. The latter is converted into g. by multiplying the number of ml. by 0·998.

The calculation is summarised in the following equation :

$$D = 100 - 99{\cdot}8(V - 0{\cdot}0055T)/W$$

where D = % dry matter
V = observed volume of distillate
T = total titre of distillate (ml. of 0·1N-NaOH)
W = weight of sample in g.

Discussion

The volatility of the lower volatile fatty acids determined by toluene distillation decreased with increasing molecular weight. This is probably because the solubilities of the volatile acids in water decrease with increasing molecular weight and consequently more of the higher acids tend to pass into the toluene during distillation. This means that the toluene method has an advantage over the oven-drying method in that the error due to volatiles is reduced. The toluene distillation apparatus was designed to accommodate 65 g. of silage. This size of sample allowed distillate volumes to be measured in the trap when the silage dry matter was within the range 11–21%. With dry matter contents higher than 21% it was necessary at the end of the determination to add a known volume of water to the trap in order to read the level in the graduated stem. It was found that a minimum time of 7 h. was necessary in order to ensure complete recovery of water from silages. The distillation, however, could be stopped overnight if necessary and completed the following day without error, provided the apparatus was not disconnected.

The volatility of lactic acid during toluene distillation was relatively low. The lactic acid recovered in the aqueous distillate was 7·1% for the pure solution and only 3·6% for the 16 silages studied. The former value is not dissimilar to that reported by Smith[8] during steam distillation.

It is clear from the results shown in Table IV that the volatiles present in the aqueous distillate obtained during toluene distillation can affect significantly the dry matter value of the silages. These volatiles range from 1·24 to 4·04% of the corrected dry matter value. Hence, in order to obtain an accurate value for silage by toluene distillation, it is necessary to determine individual volatile substances. Such determination is, however, unsuitable for routine purposes and a simplified procedure for correcting for volatiles in the distillate is desirable. A study of Table IV shows that the volatiles are mainly acidic in nature and an attempt was therefore made to relate the titratable acidity of these volatiles (determined by the Foreman method) to the volume occupied by them in the aqueous distillate. The factor for converting titratable acidity into volume of volatiles was designated 'F' and ranged from 0·00538 to 0·00615 with a mean value of 0·00555 for the 16 silages studied. The value of 'F' will vary according to the relative amounts of acids and ammonia present in the distillate. For silages with low pH, the main volatile component of the aqueous distillate will be acetic acid and the correction factor for acetic acid, namely 0·00518, may be used instead of the calculated mean value of 0·00555. The corresponding factors for propionic, butyric and lactic acids are 0·00678, 0·00843 and 0·00700, respectively. The greatest error likely to result from the use of 0·00555 as the correction factor would be an underestimate of 34% if butyric acid is the only acid present in the distillate. This is very unlikely to occur, but even if it were, the actual error in the dry matter determination would be small because of the relatively low volatility of butyric acid under these conditions of distillation.

Ammonia would cause serious interference in the calculation only when present in large amounts and if necessary this could easily be determined in the distillate. Ammonia, however, is not likely to be present in any quantity in well-preserved silages.[9] Alcohol was detected in only two of the silages. Failure to take the volume due to alcohol into account in the calculation of dry matter value resulted in the values for dry matter being underestimated by about 0·5 and 0·75% of the corrected value.

Dry matter values obtained by the toluene method using the correction factor 0·00555 in the calculation agree well with the corrected results obtained by the oven distillation method. With one exception (sample 12) the differences between the two sets of results are well below 1%.

Conclusions

The most accurate method of determining the dry matter content of silages is by the oven distillation procedure in which the volatiles are determined individually. This method is laborious for routine use, however, and the toluene distillation procedure, coupled with the Foreman titration technique, provides a simple and reliable method for determining the dry matter content of silage.

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BUFFERING CAPACITY OF HERBAGE SAMPLES AS A FACTOR IN ENSILAGE

By P. McDONALD and A. R. HENDERSON

The buffering capacity to lactic acid of a number of grass and legume samples has been determined, the results being expressed as mg. lactic acid required to lower the pH of 1 g. of dried, milled herbage samples to pH 4 (LBC values).

LBC values of samples examined range from 22 to 66, the higher results being obtained with legumes. Of 6 grass species examined, cocksfoot shows the highest LBC values.

Although the results show that there is a relationship between crude protein content and LBC, there is evidence that crude protein itself is not mainly responsible for the buffering capacity of silage crops. The importance of organic acids is discussed.

Introduction

The preservation of herbage as silage is dependent, in the ordinary process, upon the rapid acidification of the mass by lactic-acid-producing bacteria, which reduce the pH to a value usually within the range 3·8–4·2. The more quickly the formation of lactic acid can proceed, the sooner will undesirable changes be dominated. Soluble carbohydrates, lacto-bacilli, moisture, consolidation and temperature, all play an important part in the fermentation process and influence the final quality of the silage produced.

The importance of buffering capacity in A.I.V. silage making has been stressed by Virtanen[1] who stated that the amount of mineral acids which must be added to herbage in the A.I.V. process depends upon this property. He devised a rapid method for determining the amount of hydrochloric acid which must be added to the fresh crop in order to obtain the desired pH values.

It is known that plants vary in their buffering capacity and that legumes tend to be more highly buffered than grasses.[2] Wilson[3] determined the ' neutralising power ' of forage crops for organic and mineral acids and showed that leguminous crops required more acid to bring about a change of 1 pH unit than was required by non-legumes. The difficulty in ensiling legume crops has indeed been attributed to their high buffering capacity and the high protein content of these crops has been considered as the factor responsible.[4] Small[5] has stated that the following buffers have been found in plants : phosphates, carbonates, malates, oxalates, tartrates, asparagine. Potassium is commonly the most abundant base in silage crops, but calcium and magnesium are also likely to be involved.

The buffer index has been used by many workers[5] as a definite direct measure of the buffer capacity and is calculated as dB/dpH, where dB is the number of g.-equiv. per litre of acid or base required for the observed change of pH in 1 litre of buffer solution. Common[6] has made use of buffer index curves in predicting silage quality.

It is clear that if plants intended for silage have a high buffering capacity, a larger amount of acid must be produced during fermentation in order to achieve a satisfactory preservation state. There is a lack of information about the relative importance of this property during ensilage and the variations which occur between different grass and legume silage crops ; the purpose of this investigation was to study these variations in a number of silage crops and evaluate the importance of the buffering value in affecting the fermentation process in ensilage.

Experimental

In the results presented in this paper, attention is directed mainly to the relationship between buffer capacity and the amount of lactic acid required to lower the pH values of herbage macerates to pH 4. This value was adopted since silage preserved at this level is regarded as satisfactory. The method for determining the buffer capacity of grass and legume samples in these studies is described below. Determinations were carried out on both fresh and dried herbage. In every case 4·8 g. of dry matter or its equivalent as fresh material were used.

The weighed sample was macerated for 3 min. with 150 ml. of CO_2-free distilled water and the mixtures were transferred to 600-ml. beakers with further 50 ml. of water. Volumes of 0·1N-lactic acid (ranging from 5 to 35 ml.) were added with stirring and the macerates were left at room temperature with occasional stirring for 90 min. The pH was then determined with a ' Pye ' Universal pH meter and an ' Ingold ' glass electrode. The pH values were plotted against the equivalent volumes of 0·1N-lactic acid and the volumes of acid required to lower the macerate pH to 4 were interpolated from the curve. The pH values of fresh and dried macerates were also determined.

Several grass and legume samples were examined at different stages of growth. These samples were obtained from plots sited on the Bush or Boghall farms, Midlothian. In most cases, buffer determinations were carried out on fresh herbage macerates as well as on dried, milled samples. With the fresh herbage samples it was necessary to know the moisture content prior to a buffer determination and fresh samples were therefore stored in the deep freeze unit at $-13°$ until the moisture content had been measured. It had been established that deep freezing did not alter the buffer capacity of herbage samples.

Other analytical techniques used in this paper were similar to those described by McDonald *et al.*[7]

Results

Buffering capacity of different species

The time required to reach stable conditions after addition of lactic acid to grass macerates, was determined by taking pH readings at regular intervals over a 24-h. period. The results for a sample of timothy grass are shown in Table I. The pH values of these acid mixtures did not alter markedly after 90 min. and this time was used as a standard in all the buffer determinations. Fig. 1 shows the curves produced for a few of the samples examined.

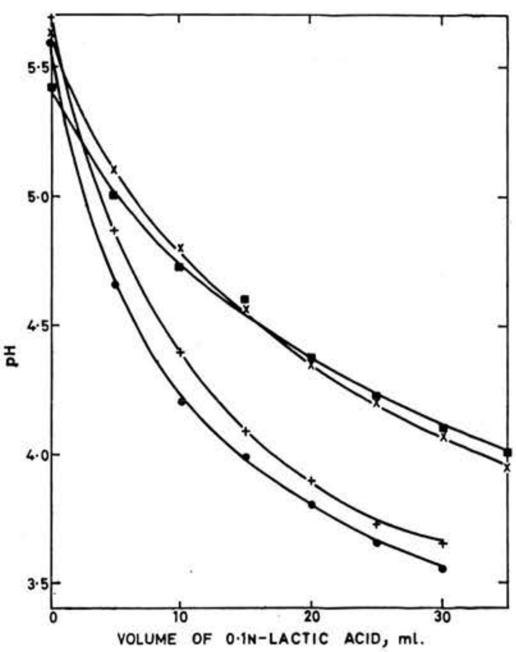

Fig. 1.—*Effect of* 0·1N-*lactic acid on pH of herbage samples* (4·8 *g. of dried material*)

● *Phleum pratense* + *Dactylis glomerata*
× *Medicago sativa* ■ *Trifolium pratense*

The interpolated buffer value at pH 4 determined with 0·1N-lactic acid and 4·8 g. of dry matter was expressed as mg. of lactic acid/g. of herbage dry matter and designated ' Lactic Buffer Capacity ' (LBC).

In order to check the reproducibility of the method, LBC values of four different herbage samples were determined in triplicate. These LBC values ranged from 21·3 to 55·2 with a mean value of 38·9 and the coefficient of variation for the 12 determinations was 0·27%.

Table I

Effect of time on the pH value of 25-g. timothy grass macerates after additions of varying amounts of 0·1N-*lactic acid*

Time interval after adding acid, h.	Fresh grass, ml. 0·1N-lactic acid: 0	10	15	20	25	30	Time interval after adding acid, h.	Dried grass, ml. 0·1N-lactic acid: 0	10	15	20	25	30
½	6·12	4·43	4·13	3·91	3·79	3·69	½	5·57	4·30	4·00	3·83	3·70	3·60
1	6·10	4·49	4·15	3·92	3·80	3·69	1	5·58	4·32	4·01	3·86	3·72	3·60
1½	6·10	4·51	4·18	3·96	3·80	3·70	1½	5·59	4·32	4·01	3·88	3·71	3·61
2	6·10	4·51	4·18	3·99	3·82	3·70	2	5·60	4·34	4·01	3·88	3·74	3·62
3	6·00	4·53	4·20	4·00	3·86	3·73	3	5·60	4·34	4·02	3·88	3·73	3·63
24	5·82	4·52	4·18	3·99	3·85	3·72	24	5·59	4·34	4·01	3·88	3·74	3·63

The LBC values for 52 dried grass and legume samples are shown in Tables II and III. On 39 of these samples LBC determinations were made on fresh as well as on dried, milled material. For the 39 pairs of samples, the mean LBC value was 35·1 for the fresh and 33·8 for the dried. The standard error of the mean difference was $\pm 0{\cdot}30$. The difference between the means for fresh and dried material was significant ($P = 0{\cdot}05$) but the convenience in handling dried milled herbage overweighs the small error incurred. The LBC values reported in Tables II and III show considerable variation ranging from 22 (*Lolium perenne*) to 66 (*Trifolium pratense*).

The LBC values were plotted against crude protein (C.P.) and the results are shown in Fig. 2, which also includes a number of other dried, milled samples. With the exception of

Table II

Buffering capacity of grasses to lactic acid

Species	Variety	Cut 1* C.P. %	Cut 1* LBC†	Cut 2 C.P. %	Cut 2 LBC	Cut 3 C.P. %	Cut 3 LBC	Cut 4 C.P. %	Cut 4 LBC
Dactylis glomerata	S143	8·0	32	16·4	38	13·0	42	10·9	38
	Danish	6·8	26	16·4	41	14·0	42	11·8	35
Phleum pratense	S51	7·7	29	11·4	32	10·9	28	9·3	24
	Scots	8·2	25	16·9	32	13·6	32	10·5	30
	Melle hay	8·4	28	15·6	28	12·0	29	8·8	26
Lolium perenne	S23	10·0	34	9·7	28	7·8	22		
	Hunsballe II	9·5	31	10·3	31				
Festuca elatior	Rhenish	6·8	25	13·5	35	10·4	29	10·2	31
	S170	6·3	26	10·9	29	9·5	29	9·3	28
Festuca pratensis	Danish	10·0	28	11·2	28	9·2	23		
	S215	8·1	29	9·1	24				
Poa pratensis	Commercial	9·3	29	13·7	28	9·9	23		
	Skandia II	8·2	25	12·6	26	9·6	23		

* Cut 1. First growth, mature, June, 1961. Cut 2, 3, 4 successive cuts of aftermath July–August, 1961
† mg. lactic acid required to lower pH to 4 in 1 g. of dried milled herbage

Table III

Buffering capacity of legumes to lactic acid

Species	Variety	C.P. %	LBC
Trifolium pratense	S151	17·7	58
	N.Z. Montgomery	18·2	66
	Essex	16·6	65
	Altaswede	18·5	65
Trifolium repens	S100	22·4	43
Vicia sativa	—	25·3	50
Vicia faba	Albyn	18·8	38
Medicago sativa	Du Puits	19·5	60

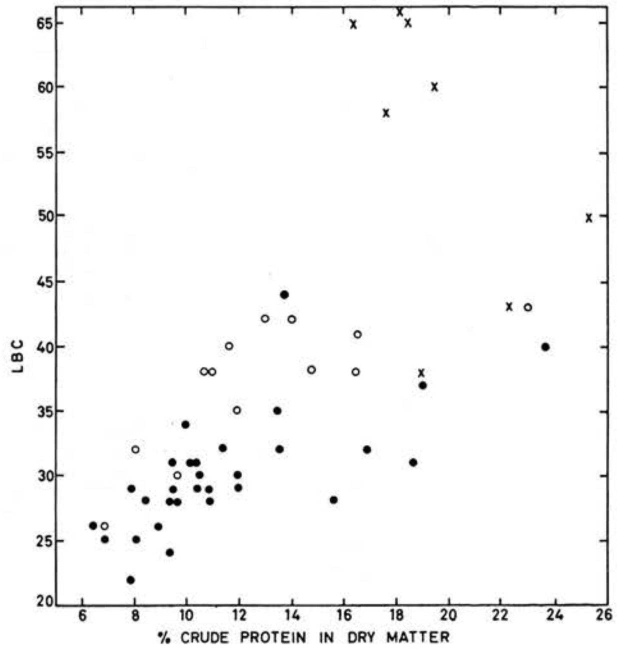

FIG. 2.—*Relationship between LBC and crude protein in grasses and legumes*
○ *Dactylis glomerata* ● other grasses × legumes

a sample of *Vicia faba*, the legumes have much higher LBC values than the grasses. Of the grasses, *Dactylis glomerata* samples have slightly higher LBC values than the other grasses with similar C.P. contents.

The mean pH values of 39 samples of (*a*) fresh and (*b*) dried, milled macerates, were respectively 5·9 and 5·8. The standard error of the mean difference was ±0·02, the pH of the fresh herbage being significantly higher (P = 0·01) than that of the dried material.

Factors influencing buffer capacity of herbage

Protein has been suggested as a possible factor influencing buffer capacity. In order to investigate this, the LBC values were determined on samples of *Lolium italicum* obtained from four plots, two of which had been heavily dressed with ammonium sulphate (9 cwt./acre). The fertiliser had been applied on 4th April, 1960, and five samples of herbage were taken from each plot over a period of 25 days. The results are shown in Table IV. In the first sample

Table IV

Effect of nitrogen fertilisation on pH and buffering capacity of Lolium italicum

Date of cutting	control						High N					
	Plot 1			Plot 2			Plot 3			Plot 4		
	C.P. %	Initial pH	LBC	C.P. %	Initial pH	LBC	C.P. %	Initial pH	LBC	C.P. %	Initial pH	LBC
11/4/60	21·6	5·65	40	21·8	5·81	43	33·8	5·09	29	35·9	5·23	33
18/4/60	17·2	5·51	38	19·9	5·43	38	35·9	4·87	29	37·4	4·89	29
25/4/60	14·6	5·61	34	16·9	5·53	34	36·3	5·20	32	37·3	5·17	32
28/4/60	12·8	5·69	37	13·5	5·65	33	33·4	5·40	38	34·4	5·30	35
4/5/60	10·6	5·63	31	11·4	5·66	32	28·7	5·31	37	28·9	5·40	38

Nitrogenous fractions (mg./100 g. of dry matter) on samples 4/5/60

	Plot 1	Plot 2	Plot 3	Plot 4
Protein N	1525	1621	3708	3865
Soluble N	171	203	884	759
NH_3-N	3	5	19	20
Amide-N	6	16	194	128

taken on 11th April, 1960, the LBC of the fertilised grass is much lower (31) than that of the control herbage (42) in spite of the high C.P. content (34·8%) of the fertilised grass compared with that of the control (21·7%). In subsequent samples the LBC of the fertilised herbage tends to increase up to the values for the control. The pH values of the fertilised herbage are substantially lower than those of the control samples. The low pH value of 4·88 for the high-N samples collected on 18th April, 1960, is noteworthy.

In further studies to determine the effect of protein on the buffering capacity of herbage to lactic acid, macerated samples of *Dactylis glomerata* were heated at 100° for 10 min. with distilled water and the buffer capacity of the protein-free filtrate determined. The LBC value of the filtrate was 69% of the value for the whole macerated sample.

LBC and silage acids

During the period 1957–59, five samples of *Lolium italicum* were ensiled without additives in the experimental silos using the techniques previously described by McDonald *et al.*[7] The silages were all well preserved and the contents of lactic and volatile acids are compared in Table V with the LBC values of the original grasses. The results are discussed below.

Table V

Dry matter composition and LBC values of grasses and the organic acids of silages (Lolium italicum)

Variety	Date of cutting	Grass							Silage			
		Dry matter %	Total N %	Protein N %	Non-protein N %	Soluble carbo-hydrates, %	LBC	pH	Acids: Lactic %	Acetic %	Pro-pionic %	Butyric %
S22	5/7/57	19·2	3·00	2·66	0·34	15·1	31	3·8	8·7	1·8	nil	nil
S22	3/10/57	19·9	3·04	2·73	0·31	16·1	37	4·3	10·4	1·6	nil	nil
Irish	30/7/58	14·9	1·92	1·71	0·21	17·0	30	3·7	11·5	2·1	nil	0·18
S22	14/10/58	15·6	2·21	—	—	16·2	44	4·1	11·5	1·8	nil	0·20
S22	7/5/59	21·5	3·80	3·05	0·75	19·5	40	4·0	17·9	2·8	nil	nil

Discussion

The LBC values obtained with dried, milled samples were significantly lower than those for fresh materials. The differences, however, were not great and so the LBC values can conveniently be determined on dried, milled samples as a routine procedure.

A number of workers[8–10] have commented on the difficulty of ensiling high-protein crops by the ordinary method and have indicated the importance of buffer capacity in ensilage. The results of the present studies suggest that there is a relationship between crude protein (C.P.) content and buffering capacity in grasses, although the relationship varies with species. Legumes tend to be more highly buffered than grasses at similar C.P. contents. Although this relationship exists, it is unlikely that C.P. is mainly responsible for the buffer capacity since herbage heavily fertilised with ammonium sulphate could have a relatively low buffer value as well as a high C.P. content.

The buffering capacity of plants has also been attributed to the potassium, calcium and magnesium salts of organic acids.[5] Hirst & Ramstad[11] studied the organic acid content of S-24 *Lolium perenne* and found that the principal acids present were malic, quinic, citric and smaller amounts of succinic. Although no organic acid determinations were made in this work, it is interesting to note that other studies have shown that plants supplied with ammonium-nitrogen contain much lower concentrations of acids than do similar plants grown with nitrate-nitrogen.[12] It is possible that the depression in buffer capacity of herbage heavily fertilised with ammonium sulphate reported here may have been associated with a reduction in the organic acid fraction.

From the results given in Table V it is clear that the buffer capacity and/or neutralising value increases during ensilage. If lactic acid production occurred almost immediately, then a lactic acid content of 3–5% of the dry matter should theoretically be capable of preserving grass at the pH 4 level. That this does not occur in practice suggests that chemical changes which increase the buffer capacity take place during the early stages of the fermentation process.

These changes may be due to a number of factors. Considerable breakdown of protein to amino-acids occurs in the early stages of ensilage. Amino-acids themselves are unlikely to contribute to any extent to the buffering capacity of the silage within the pH range 4–6, but decarboxylation of amino-acids with the production of basic residues may occur. Under these conditions lactic acid may be neutralised by the bases so formed.

The changes in organic acid content during ensilage may also have an important effect in increasing both the neutralising power and buffering capacity of the herbage. Hirst & Ramstad[11] showed that there was a rapid and complete breakdown of malic and citric acids during ensilage. Whittenbury[13] has examined the citrate- and malate-dissimilating activities of different types of lactic acid bacteria isolated from silage and fresh grass. Breakdown varied, some types having no activity, some dissimilating these salts vigorously only in the presence of a sugar and some only in the absence of a sugar. Dissimilation was indicated by production of carbon dioxide, rise in pH and the formation of neutral products. This rise in pH occurring in the early stages of ensilage has been noted by other workers.[7, 14]

The liberation of cations by decarboxylation of citrates and malates will increase the neutralising power of the ensiled herbage, and lactates and acetates are liable to be formed. The presence of these weak acid salts will themselves act as a buffer system to developing lactic acid and will increase the buffer capacity of the silage. As Whittenbury[13] has stated, the initial organic acid content of the herbage and the types of bacteria which develop during ensilage are clearly of vital importance in deciding the amount of lactic acid which must be produced to achieve stable conditions.

It follows that if the secondary changes causing an increase in buffering capacity could be restricted or inhibited, then a smaller amount of lactic acid would need to be produced in order to preserve the silage at a satisfactory pH level. Further studies along these lines are in progress.

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6

FERMENTATION STUDIES ON WET HERBAGE

By P. McDONALD, A. C. STIRLING, A. R. HENDERSON and R. WHITTENBURY

Two experiments are described in which the fermentation changes in ensiled wet herbage were studied. In the first experiment *Lolium italicum*, containing 19·6% of soluble carbohydrate, was used in four silos. Water was added in two silos during filling to reduce the dry matter content from 21·5% to 17·0%. Although all four silages were well preserved the gaseous losses occurring in the 'wet silages' were almost three times those obtained from the drier materials. The addition in one treatment of a small amount of soil (0·1%) during filling did not affect adversely the fermentation changes.

In the second experiment, *Dactylis glomerata* containing 6·2% of soluble carbohydrate was ensiled with and without the addition of water. In this experiment 2% molasses was added to two silos as an additional treatment. The resultant silages were not well preserved although the addition of molasses and water produced a silage of lower pH value than when water alone was added.

Introduction

A number of workers have shown that the moisture content of an ensiled crop has an important effect upon the ensilage process. The amount of moisture present affects the rate of fermentation and total bacterial count.[1] Wieringa[2] has demonstrated that the pH-tolerance of butyric acid bacteria decreases with increasing osmotic pressure, a property of herbage related largely to the moisture content. Ensiling wilted herbage causes a delay in bacterial multiplication[3] and anaerobes tend to be reduced in wilted material.[4, 5]

The importance of the moisture content of silages on dry matter intake by stock has also been stressed[6] but the evidence is that intake variations are more likely to be related to fermentation processes associated with wet herbage.[7]

Watson & Nash[8] have defined a 'wet' silage as one made from herbage containing more than 80% of moisture and such silages are frequently made in farming practice.[9] Although it is clear that an excess of moisture is deleterious to the ensilage process, there is still a lack of information about the actual effects of moisture on fermentation and nutrient losses during ensilage.

This paper presents the results of two separate experiments designed to study the effect of increasing the moisture content by spraying a known volume of water on to herbage at the time of ensiling. In the first, a cut of Italian ryegrass (*Lolium italicum*) of high soluble carbohydrate content was used; in the second, cocksfoot (*Dactylis glomerata*) of low soluble carbohydrate content was ensiled.

Experiment 1

Procedure

The silo unit used in this investigation consisted of four metal silos (153 cm. diam. × 182 cm. high), each having a maximum capacity of about 1000 kg. of fresh herbage. The silos were suspended from a weighing device which enabled daily measurements of weight changes to be recorded. Each silo was fitted with ten thermocouples which could be sited at different levels within the herbage during filling. A full description of the silo unit and the techniques of sampling, filling, methods of analysis, digestibility and bacteriological methods have been described elsewhere.[10]

Preliminary tests showed that the four silos, filled under identical conditions with *Lolium italicum*, yielded similar results for the day-to-day changes, dry matter losses and nutritive values of the silages.[10]

In the first experiment *Lolium italicum* was cut with a mower on 7th May, 1959, and ensiled the same day. The herbage was well consolidated by tramping during filling. In two silos (B and D) sufficient water was added from a pressure spray to reduce the dry matter content of the grass from 21·5 to 17·0%. The water was well mixed with the herbage during filling.

In order to study the possible adverse effects of soil contamination, a 1-cm. layer of soil (25 kg.) was placed in the bottom of silos C and D, care being taken to avoid blocking the effluent drainage pipe leading from the centre of the silo. In each of these two silos, 1 kg. of soil was also distributed throughout the herbage during filling. The soil had been obtained from the base of a farm silo in which silage of poor quality had been made the previous year. The treatments were as follows:

Silo A grass (910 kg.)
Silo B grass (718 kg.) + water (192 kg.)
Silo C grass (909 kg.) + soil (1 kg.)
Silo D grass (719 kg.) + water (190 kg.) + soil (1 kg.)

Each silo was graduated from the bottom in cm. and during filling the ten thermocouples were placed initially at the following levels:

1. 35 cm. central	6. 95 cm. outer
2. 35 cm. outer	7. 125 cm. central
3. 65 cm. central	8. 125 cm. outer
4. 65 cm. outer	9. 145 cm. central
5. 95 cm. central.	10. 145 cm. outer

The thermocouples placed in the outer position were situated about 20 cm. from the silo wall. Daily temperature readings were taken throughout the period of the experiment.

The silos and contents were weighed before they were covered with polythene sheeting, wooden compression discs and stone blocks. The total consolidation applied to each silo was 680 kg. which corresponded to a pressure of 37 g./sq. cm.

To restrict the drainage from silos B and D, the effluent taps were closed and only opened after 14-day intervals for a 2-h. period, until the 90th day when the taps were kept open until the end of the ensiling period. The effluent taps in silos A and C remained open throughout the experiment. The silos were emptied on 25th August, 1959, 111 days after filling, and the contents were sampled for subsequent analysis and digestibility studies. Detailed carbohydrate analysis using paper chromatographic separation was made on all samples.[10] Dry matter corrections were made for volatile losses occurring during drying by an oven distillation technique.[11] Digestibility studies were carried out in triplicate on Cheviot wether sheep.[10]

At the time that the silos were filled, the bacterial content of the fresh herbage was examined. A series of 'tube silos' (each containing 50 g. of the fresh material) was set up in the laboratory in order to provide material for an investigation of the bacterial changes during the first week after ensiling. Fifty-g. samples of herbage, or silage, or in the case of the laboratory silages the contents of one tube, were made up to 300 g. with sterile water and macerated. Suitable dilutions of the macerate were plated on glucose yeast agar for a count of viable organisms. A count of lactobacilli was obtained from acetate agar and of Gram-negative bacteria from ammonium lactate agar. Tubes of inoculated medium covered with an agar seal were used for estimating the number of anaerobes. The media and the methods used have been fully described elsewhere.[10] One set of laboratory silages was held at 30° and two sets at room temperature. One set of the latter was made up with the addition of soil and water, a treatment comparable with that used for the large silo D.

Results

Volume changes

The original volume of the ensiled herbage was 3·02 cu. m. but this fell rapidly in all four silos until by the fifth day the volume had been reduced to half the original value. The changes in volume thereafter followed a similar pattern and at the end of the ensiling period were A 1·0; B 0·81; C 1·05 and D 0·90 cu. m.

Composition

The chemical composition of the grass and of the four silages made from it are given in Table I. The silages (Table II) excluding surface waste, were well preserved (pH 4·0–4·1) and contained relatively large amounts of lactic acid. Butyric acid was present in silages B, C and D, the highest amount being present in silage D. In all four silages small amounts of formic acid were present, while the acetic acid values were similar. The Italian ryegrass cut at a young leafy stage of growth was high in nitrogen and low in crude fibre content. The ' wet ' silages contained slightly higher amounts of volatile nitrogen than did the drier silages.

Table I

Composition of grass and silages (% of true dry matter)

	Experiment 1					Experiment 2				
	Grass	Silages				Grass	Silages			
		A	B	C	D		A	B	C	D
True dry matter	21·47	21·33	17·03	21·44	16·56	17·52	16·17	13·75	16·95	15·02
Organic matter	90·4	88·5	88·1	87·8	88·2	90·3	89·2	90·2	88·9	90·3
Crude protein	23·7	24·9	25·5	25·1	24·7	20·0	23·2	20·8	22·3	20·3
Ether extract	3·7	5·3	5·4	5·3	5·2	3·7	4·7	5·6	5·7	5·6
Crude fibre	16·1	17·6	19·7	17·9	19·4	25·4	31·6	32·3	29·4	29·9
N.F.E.‡	46·9	40·7	37·4	39·5	39·0	41·3	29·8	31·5	31·6	34·5
Total N	3·80	3·98	4·09	4·02	3·94	3·20	3·71	3·33	3·57	3·24
Protein N	3·05	1·44	1·56	1·47	1·43	2·83	1·12	1·06	1·26	1·44
Non-protein N	0·75	2·54	2·53	2·55	2·51	0·37	2·59	2·27	2·31	1·80
Volatile N	nil	0·37	0·44	0·39	0·44	0·02	1·20	0·85	0·88	0·47
Total sugars	13·7	2·5	0·8	2·0	1·1	4·6	0·3	0·3	0·5	0·5
Sucrose	8·4	—	—	—	—	1·8	—	—	—	—
Glucose	2·2	—	—	—	—	0·9	—	—	—	—
Fructose	1·9	—	—	—	—	1·1	—	—	—	—
Oligosaccharides*	1·2	—	—	—	—	0·8	—	—	—	—
Fructosan	5·9	0·11	0·07	0·12	0·08	1·6	0·06	0·05	0·11	0·14
Total hemicelluloses	11·2	8·7	9·6	8·4	9·3	16·4	14·3	15·4	13·6	0·14
Galactan	0·6	0·8	0·9	0·7	0·9	1·2	0·9	1·0	0·9	1·1
Araban	3·2	1·6	2·4	1·5	2·2	3·2	2·4	1·9	2·6	3·2
Xylan	7·4	6·3	6·3	6·2	6·2	12·0	11·0	12·5	10·0	9·7
Cellulose	18·3	19·4	22·2	19·6	21·8	27·4	34·0	34·6	31·8	32·3
Lignin	4·6	4·1	4·3	3·8	4·2	5·9	7·5	7·5	6·7	7·3
Lactic acid	—	17·9	18·7	17·6	18·9	—	0·07	0·09	0·11	0·23
Formic acid	—	0·10	0·13	0·15	0·08	—	nil	nil	nil	nil
Acetic acid	—	2·86	2·83	2·87	2·93	—	3·72	4·16	2·44	2·89
Propionic acid	—	nil	0·20	0·06	nil	—	1·45	nil	nil	nil
Butyric acid	—	nil	0·22	0·19	1·02	—	3·63	3·72	3·46	1·83
pH†	—	4·0	4·0	4·0	4·1	—	5·4	5·0	5·2	4·6

* Excluding sucrose
† pH and true dry matter determined on fresh material
‡ Nitrogen-free extractives

Table II

pH values of silage samples

Sample		Experiment 1				Experiment 2			
		A	B	C	D	A	B	C	D
1.	Surface waste	5·0	4·9	4·5	4·9	6·8	6·2	5·9	7·0
2.	Top layer	4·0	4·0	4·0	4·1	5·4	5·2	5·4	5·1
3.	—	4·0	4·0	4·0	3·9	5·4	5·0	5·2	4·4
4.	—	4·0	4·0	4·0	4·0	5·4	5·0	5·3	4·2
5.	—	3·9	4·0	4·0	4·1	5·3	5·0	4·7	4·4
6.	—	4·0	—	4·2	—	—	—	—	—
7.	Bulked	4·0	4·0	4·0	4·1	5·4	5·0	5·2	4·6

The soluble carbohydrate (sugar + fructosan) content of the grass was relatively high (19·6%) and the main sugar present was sucrose. Cellulose was determined by the method of Crampton & Maynard[12] and includes some pentosan and nitrogen. The silages contained relatively more cellulose and crude fibre than the grass, the ' wet ' silages containing the highest amounts.

The pH values of the effluents from the four silos fell to 4·5 by the 6th day and declined steadily to pH 4·2 by the 11th day. The values then remained fairly constant (pH 4·0–4·2) until the end of the ensiling period.

Temperature changes

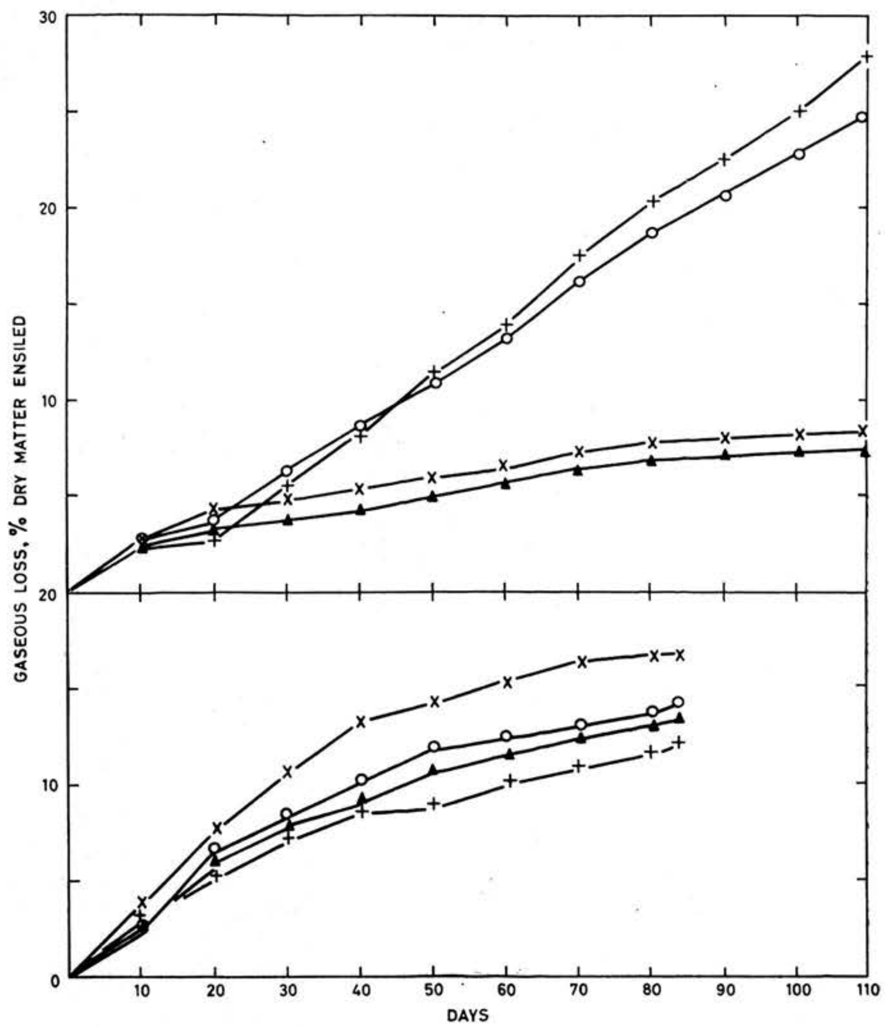
The maximum mean temperatures in all four silos were similar (19°) and were recorded towards the end of the ensiling period, coinciding with a similar ambient temperature.

Losses

The total weights of silages removed from the silos at the end of the ensiling period were 826, 689, 833 and 733 kg. for silos, A, B, C and D respectively. The losses due to surface waste were low and amounted to 1·6, 1·9, 1·4 and 2·6% of total silage for A, B, C and D respectively. Waste material was analysed separately and the results were taken into account in calculating losses.

The gaseous losses are shown in Figs. 1 and 2. The gaseous losses occurring in the 'wet' silages were about three times those from the drier silages.

The detailed losses of individual constituents are given in Table III. The dry matter losses in the 'wet' silages were much higher than in the drier silages and this could not be accounted for entirely in terms of effluent. The gaseous dry matter losses from silos A, B, C and D were 6·4, 15·1, 5·5 and 14·7% respectively. Although almost complete recoveries of nitrogen



Gaseous losses as % of dry matter ensiled

FIG. 1 (above) Expt. 1, FIG. 2 (below) Expt. 2
× = silo A ○ = silo B △ = silo C + = silo D

Table III

Percentage losses during ensilage

	A		B		C		D	
	Total	Effluent	Total	Effluent	Total	Effluent	Total	Effluent
Experiment 1								
Total fresh	9·2	7·3	24·3	19·2	8·4	6·8	19·3	13·5
Dry matter	9·8	3·2	23·9	8·8	8·5	3·0	21·2	6·5
Crude protein	5·6	4·2	18·3	9·6	3·2	3·6	18·0	9·6
Ether extract	+29·2	—	+12·3	—	+31·9	—	+10·5	—
Crude fibre	1·3	—	6·5	—	+1·9	—	5·2	—
N.F.E.	21·7	—	39·4	—	23·0	—	34·5	—
Total N	5·6	4·2	18·3	9·6	3·2	3·6	18·0	9·6
Protein N	57·4	—	61·1	—	55·8	—	63·1	—
Sugars	83·5	9·5	95·6	3·2	86·6	8·8	93·7	1·7
Fructosan	98·4	—	99·1	—	98·2	—	98·9	—
Cellulose	4·4	—	7·8	—	1·7	—	6·4	—
Hemicelluloses	29·9	—	34·7	—	31·4	—	34·7	—
Galactan	+20·5	—	+14·0	—	+6·8	—	+17·2	—
Araban	54·9	—	42·8	—	57·1	—	45·7	—
Xylan	23·2	—	35·1	—	23·3	—	34·0	—
Lignin	19·8	—	28·8	—	24·4	—	28·0	—
Water	9·0	8·4	24·2	21·3	8·3	7·9	18·9	14·9
Experiment 2								
Total fresh	19·0	16·1	31·5	30·4	12·5	10·0	32·6	30·4
Dry matter	25·2	6·2	24·8	9·4	23·9	5·1	23·7	11·2
Crude protein	13·1	13·6	21·0	18·1	3·4	7·1	16·9	16·2
Ether extract	3·9	—	+15·2	—	+34·9	—	+26·1	—
Crude fibre	7·0	—	4·3	—	+0·8	—	2·9	—
N.F.E.	46·1	—	42·5	—	42·8	—	40·8	—
Total N	13·1	13·6	21·0	18·1	3·4	7·1	16·9	16·2
Protein N	70·4	—	71·8	—	61·0	—	57·9	—
Sugars	94·5	0·6	95·8	2·5	95·6	10·0	90·7	8·6
Fructosan	97·2	—	97·7	—	93·9	—	93·0	—
Cellulose	7·2	—	5·0	—	+1·0	—	2·8	—
Hemicelluloses	34·8	—	29·6	—	27·9	—	29·8	—
Galactan	47·6	—	42·3	—	33·7	—	25·0	—
Araban	42·8	—	54·8	—	71·2	—	16·3	—
Xylan	31·3	—	21·6	—	28·0	—	33·8	—
Lignin	5·2	—	4·5	—	0·5	—	+1·4	—
Water	17·6	18·3	32·5	33·4	11·0	10·9	34·0	33·4

were obtained from the silos containing the drier silages, the results suggest that about 8% of nitrogen was lost in gaseous form from the ' wet ' silages. About 9% of sugars were lost in the effluent produced from the ' wet ' silages whereas the effluent losses from the drier materials were comparatively low. About one-third of the hemicelluloses were unaccounted for. The apparent gain in galactan suggests that some contamination had occurred during chromatographic separation. It is doubtful if this is important, however, since the initial galactan content of the grass was very low.

Nutritive value

The digestibility coefficients and digestible nutrients of the grass and silages are given in Table IV. The digestibility values reported are the mean values obtained from trials carried out in triplicate with Cheviot wethers. The results indicate that there was very little difference in digestibility between the grass and silages.

Bacteriological studies

The content of one tube from each set of the laboratory-made silages was examined 1, 4 and 8 days after filling. The bacterial counts and pH values are given in Table V.

In silage held at 30°, the development of lactic acid bacteria and of anaerobes was more rapid than in the silage held at room temperature. The pH did not drop rapidly and was not

Table IV

Percentage digestibility (D) and percentage of digestible nutrients (DN) in true dry matter

	Grass		Silages							
			A		B		C		D	
	D	DN	D	DN	D	DN	D	DN	D	DN
Experiment 1										
Organic matter	84·4	76·3	85·1	75·1	85·0	74·9	83·9	73·7	85·9	76·0
Crude protein	82·0	19·4	83·9	20·7	83·7	21·3	83·0	20·8	84·9	21·1
Ether extract	61·9	2·3	81·6	4·2	82·1	4·4	79·2	4·2	81·6	4·3
Crude fibre	87·3	14·1	90·9	17·6	91·4	18·0	90·0	16·1	91·3	16·1
N.F.E.	86·3	40·5	83·5	32·6	82·8	31·0	82·5	32·6	84·6	34·4
S.E.	—	72·5	—	72·0	—	71·7	—	71·0	—	73·4
T.D.N.	—	79·2	—	80·4	—	80·2	—	79·0	—	81·3
Experiment 2										
Organic matter	75·7	68·4	74·2	66·2	75·8	68·4	78·5	69·8	74·1	66·9
Crude protein	75·4	15·1	80·1	18·6	81·4	16·9	82·8	18·5	77·6	15·8
Ether extract	45·1	1·7	54·4	2·6	59·1	3·3	60·8	3·5	59·6	3·3
Crude fibre	79·9	20·3	82·9	26·2	82·5	26·6	84·1	24·7	83·8	25·1
N.F.E.	75·5	31·2	63·6	19·0	67·9	21·4	73·7	23·3	64·3	22·2
S.E.	—	62·1	—	58·4	—	61·9	—	63·6	—	59·8
T.D.N.	—	70·4	—	69·7	—	72·3	—	73·4	—	66·7

T.D.N. = total digestible nutrients S.E. = starch equivalent

below 4·5 after 8 days although the bacterial activity was greater than in the silage held at 22°. Lactobacilli were not detected even in 1 ml. of the undiluted macerate of the fresh herbage, and 24 h. after ensiling they had shown little development. A comparison of the two sets of silages held at room temperature shows that the addition of soil and water resulted in an increased development of bacteria of all types.

Samples from the large experimental silos all had low pH values and the bacterial populations consisted almost entirely of lactobacilli. The silage containing water and soil had more anaerobes than that containing soil alone. The fresh herbage contained so few lactobacilli that the small quantities used in the laboratory silages did not provide a sufficient inoculum to ensure a rapid fermentation and low pH in the first week. The consolidation of the material in the large silos probably resulted in the distribution of the few lactobacilli that were present on the fresh grass, so giving rise to a more active fermentation than occurred in the tube silos.

Experiment 2

Procedure

The second experiment was carried out in 1960 by a similar procedure to that in Expt. 1, with cocksfoot (*Dactylis glomerata*) instead of Italian ryegrass. In this experiment the quantities of grass in each silo were similar and a greater volume of water was added than in the previous experiment. The treatments were as follows:

Silo A grass (762 kg.)
Silo B grass (750 kg.) + water (300 kg.)
Silo C grass (750 kg.) + molasses solution (22·5 kg.)
Silo D grass (757 kg.) + water (300 kg.) + molasses solution (22·5 kg.)

The molasses solution consisted of 15 kg. of molasses diluted with 7·5 kg. of water and this was sprayed on to the grass from a pressure spray during filling. The molasses contained dry matter 70%, total N 0·27% and sugars 45·6%. Soil was not added in this experiment. The grass was cut with a mower on 19th May, 1960, and ensiled the same day. The contents of each silo occupied 3·02 cu. m. and the consolidation weight applied was 680 kg. as in the previous experiment.

Effluents were collected daily, although drainage from silos B and D was restricted to 5·5 l./day from the 1st to the 22nd day after ensiling, reduced to 2·75 l./day from the 23rd to the 77th day, and from the 78th day to the end of the ensiling period the silos were allowed to drain freely. The silos were opened on 10th August, 84 days after filling.

The chemical analysis, nutritional and bacteriological studies were carried out as described under Expt. 1.

Table V

pH values and bacterial counts from grass and silage

		Bacterial count (millions/g. dry wt.) on glucose yeast agar	acetate agar	lactate agar	Count of anaerobes	pH
Experiment 1						
Fresh grass		67	not detected	4·1	—	—
Laboratory-made silage						
Held at 30°	1 day	70	9·3	14	<0·01	6·7
	4 days	890	1350	7·8	1·3	5·0
	8 ,,	1170	930	4·1	0·13	4·5
	310 ,,	—	—	—	—	4·5
Room temperature	1 day	100	<0·01	14	<0·01	6·8
	4 days	50	49	0·9	<0·01	6·7
	8 ,,	1070	890	0·4	<0·01	5·9
	310 ,,	—	—	—	—	4·2
Room temperature (soil and water added)	1 day	85	0·03	6·8	0·03	6·9
	4 days	1780	260	11	<0·01	6·4
	8 ,,	1510	955	2·7	<0·01	4·8
	310 ,,	—	—	—	—	4·0
Large silos						
Control, A		860	770	—	0·65	—
Water, B		270	510	—	0·11	—
+ Soil, C		510	520	—	0·03	—
+ Soil + water, D		660	470	—	0·41	—
Experiment 2						
Fresh grass		0·3	<0·01	<0·3	—	6·5
Laboratory-made silage at 30°						
Untreated, held	2 days	1510	61	<0·3	—	6·3
	8 ,,	427	316	—	—	6·0
	88 ,,	4·3	5	<0·01	72	5·1
Wet, held	2 ,,	11500	6030	11	—	5·8
	8 ,,	398	724	—	—	5·2
	88 ,,	7·6	10	<0·01	32	4·8
Molassed	2 ,,	3980	2140	126	—	5·4
	8 ,,	794	933	—	—	5·2
	88 ,,	1·7	0·7	<0·01	320	5·3
Wet, molassed	2 ,,	16600	15100	13	—	5·0
	8 ,,	1050	692	—	—	5·2
	88 ,,	63	72	<0·01	72	5·4
Large silos after 84 days						
Control, A		140	600	—	40	—
Wet, B		1200	1200	—	4·7	—
+ Molasses, C		900	1500	—	51	—
+ Water + molasses, D		1900	3600	—	0·11	—

Results

Volume changes

The original volume of herbage in the silo (3·02 cu. m.) did not fall to half this value until the 12th day after ensiling. The final volumes of silage at the end of the experiment were A 0·81; B 1·00; C 1·00 and D 1·00 cu. m.

Composition

The original dry matter content of the cocksfoot was 17·52% and this was reduced at the time of filling to 12·51 and 13·26 in silos B and D respectively. The dry matter content of the molassed grass was 18·38%. The composition of the herbage and silage dry matter is given in Table I and the pH values of the silages in Table II.

The silages were not well preserved and contained only small amounts of lactic acid. Silage D had the lowest pH value (4·6), the others all being above pH 5·0. Appreciable amounts of butyric acid were present in all silages.

The soluble carbohydrate content of the cocksfoot was low (6·15%) compared with that present in the ryegrass. The cellulose, hemicelluloses, lignin and crude fibre contents of the cocksfoot were much higher than those of the ryegrass of the first experiment.

The graph in Fig. 3 shows the pH pattern of the effluents. Effluent was not produced from silo A until the 17th day when the pH value was 5·7. The pH results for silo A shown on the graph prior to the 17th day were obtained from electrode probes inserted through the bottom port. In silo A the effluent pH remained high throughout the experiment, but in silos C and D it fell below 4·0 on the 30th day and then rose. In silo B the pH fell to about pH 4, then rose to 5·0 on the 44th day and remained about this level.

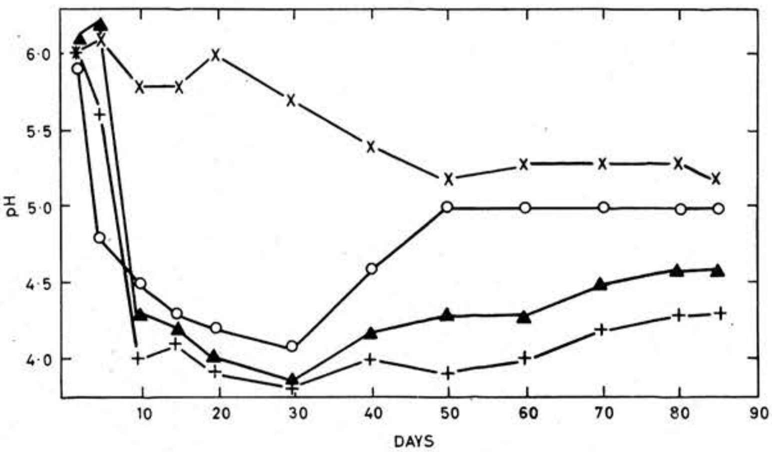


FIG. 3.—*Effluent pH values*
(Expt. 2)
× = A △ = C
○ = B + = D

Temperature changes

The maximum mean temperatures in the four silos occurred between the 18th and the 20th days. These were A 21°; B 20°; C 21° and D 19°.

Losses

The total weights of silage removed from the silos on the 84th day were: A 618; B 719; C 676 and D 727 kg. The losses due to surface waste, expressed as a percentage of the total silage removed were: A 8·3; B 2·0; C 6·9 and D 1·3. The gaseous losses are summarised in Fig. 2 and the detailed losses of individual constituents are given in Table III. The losses of dry matter from all four silos were relatively high, ranging from 23·7 to 25·2%. The effluent dry matter losses were greatest in the two 'wet' silages. The gaseous dry matter losses in the four silages were A 19; B 15·4; C 18·8 and D 12·5%.

Nutritive value

The digestibility coefficients and digestible nutrients for the cocksfoot and silages are given in Table IV. The digestibility of the organic matter ranged from 74·1% (silage D) to 78·5% (silage C). The digestible crude protein (D.C.P.) values ranged from 16·7% (silage A) to 18·9% (silage B). The Starch Equivalent values for the cocksfoot grass and silages were considerably lower than the values obtained for the ryegrass of the previous experiment.

Bacteriological studies

Four sets of laboratory silages were made. Treatments of the grass were comparable to those of the large silos, all tubes being held at 30°. The count of bacteria on the fresh herbage (Table V) was low but in this instance a count of lactobacilli was obtained. Only a few colonies, however, developed from 1 ml. of undiluted macerate. Multiplication of bacteria was rapid and after 2 days the number was highest in the ' wet-molassed ' material. Moisture showed a greater stimulatory effect than molasses. An appreciable count of anaerobes was found at the 88-day examination. The pH fell below 5·0 only in the ' wet ' silage examined after 88 days. Putrefactive odours were noticed when many of the tubes were opened, especially with the watered silages. In the large experimental silos the highest bacterial counts and the lowest pH values were obtained in the ' wet-molassed ' silage.

Discussion

During ensilage, dry matter may be lost from the silo either in the effluent or by gaseous production. It is well known that effluent losses are to a large extent related to effluent volume and this is borne out by the results of these two experiments. The gaseous dry matter loss is a result of both plant and bacterial enzyme action. With wet herbages, aerobic respiration is likely to be reduced quickly and most of the gaseous production, thereafter, will be a result of bacterial activity. The main gaseous product is carbon dioxide and this is produced from the breakdown of soluble carbohydrates, organic acids and in some cases amino-acids. Decarboxylation of histidine and tyrosine may produce histamine and tyramine. Silages B and C (Expt. 2) were examined for these two amines and they were found to be present in both in similar amounts, the quantities being histamine about 250 mg. and tyramine 1750 mg. per kg. of silage dry matter. The possible toxic effects of these amines to the animal have been discussed by Macpherson.[13]

The most outstanding feature of the first experiment, with Italian ryegrass, was the relatively high losses of dry matter associated with the wet silages, compared with the drier silages. The gaseous dry matter losses from the wet material were about three times as great as those from the drier silage. This higher loss can be associated with an increased bacterial activity in the wet material. Although it is likely that most of the gaseous matter lost from the wet silages was in the form of carbon dioxide, it is clear that some nitrogenous compounds were also lost in gaseous form, since about 8% of total nitrogen was unaccounted for.

The results of the first experiment indicate that well-preserved silage may be made from herbage of low dry matter content (17%) provided the ensiled material contains adequate amounts of soluble carbohydrates. The initial soluble carbohydrate content of the ryegrass was relatively high (19·6%) and the large amount of lactic acid and lactates (17·6–18·9% as lactic acid) in the well-preserved silages suggests that most of the soluble carbohydrates were necessary in order to preserve the material at the pH 4–4·1 level. In previous studies[14] it has been shown that a maximum lactic acid content of about 6% is sufficient to overcome the buffering properties of grass crops and reduce the pH value to 4·0. The high lactic acid content of the silages obtained in Expt. 1 indicates that the material had developed considerable buffering and/or neutralising properties. It is possible that the few lactic acid bacteria which must have been present on the original herbage were of the type which could influence the buffer capacity of the silage. Whittenbury[15] has demonstrated that different types of lactic acid bacteria vary in their action on organic acids in the presence of sugars.

Associated with high losses of dry matter was a relative increase in cellulose concentration. This increase was also reflected in the crude fibre fraction. About 30% of hemicelluloses were unaccounted for in these experiments. Detailed chromatographic analysis of the silage sugars was not carried out, but it is known from previous studies that pentoses are produced during ensilage.[10] The presence of hemicelluloses in both *Lolium italicum* and *Dactylis glomerata* has been demonstrated by Dewar.[16] Since pentoses are a readily available source of carbohydrate to lactobacilli, the production of pentoses from hemicelluloses during ensilage is likely to result in an increase of lactic and acetic acids.

The ' lignin ' results are difficult to explain and may be misleading because of the crude method of analysis.

In spite of the differences in losses between the wet and control silages, the digestibility and nutritive value of the four ryegrass silages were similar and, apart from the digestible carbohydrate fraction, did not differ markedly from the original herbage.

The addition of soil had little effect upon the fermentation processes.

In the second experiment, with cocksfoot, the fermentation changes in both the wet and control silages were very different from those observed in the ryegrass experiment. Here a major limiting factor was clearly the low content of soluble carbohydrates (6·2%); an additional factor was the lower dry matter content of the original herbage (17.5%). The cocksfoot was also more fibrous than the ryegrass, with appreciably larger amounts of cellulose, hemicelluloses and 'lignin' and it also showed a higher lactobacillus count.

In the cocksfoot experiment, a butyric acid type of fermentation occurred and in only one silo (D) was the final pH value of the contents below 5·0. Even the addition of 2% of molasses to the fresh herbage was insufficient to bring about a satisfactory type of fermentation, although in the wet-molassed treatment the gaseous dry matter losses were reduced to 12·5% compared with 15·4% in the wet herbage. The addition of both water and sugar appeared to encourage lactobacilli to develop, but even though sufficient acid was produced to reduce the pH value of the effluent to about 4·0 by the 30th day, conditions in the silo were such that this value increased eventually to 4·3. Although the pH of the effluent can be taken as a guide to the fermentation reaction which occurred within the silo, it is difficult to relate this value to the pH of the mass of silage as a whole, since the effluent is not a representative extract of the silage but only that of the bottom layers. This is illustrated from a comparison of the data given in Table II with the final effluent pH values shown in Fig. 3.

It is possible that, if very wet herbage is to be ensiled in practice, then the addition of molasses may be beneficial, although the generally recommended level of 2% to the fresh herbage may be inadequate to ensure a satisfactory lactic acid content.

The gaseous dry matter losses from the drier cocksfoot silages were higher (19%) than those from the wet silages. This may have been the result of increased respiration losses rather than bacterial fermentation.

In these experiments the herbages were not lacerated or bruised and the fibrous nature of the cocksfoot may have affected the release of juices, which would influence the rate of subsequent fermentation. The effects of laceration on fermentation changes is being studied.

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THE HYDROLYSIS OF GRASS HEMICELLULOSES DURING ENSILAGE

By W. A. DEWAR,* P. McDONALD and R. WHITTENBURY

The production of reducing sugars, resulting from the incubation of a hemicellulose prepared from *Lolium perenne* (perennial ryegrass), with enzymes extracted from *Lolium perenne, Lolium italicum* (Italian ryegrass) and *Dactylis glomerata* (cocksfoot) was measured over a range of temperature, pH and time. Each of the three enzymes had an optimum pH of 6 but the optimum temperature ranged from 30° to 43°. There was a significant interaction between high temperature and low pH, both tending to suppress enzyme activity. Appreciable amounts of reducing sugars were also produced from hemicelluloses by acid hydrolysis (pH 4) over a 90-day period.

Attempts to grow a number of strains of lactic acid bacteria using hemicellulose as an energy source were unsuccessful. The importance of these findings on the ensilage process is discussed.

Introduction

In the ordinary silage process, lactic acid bacteria convert soluble carbohydrates mainly to lactic and acetic acids.[1] For satisfactory levels of acids to be produced, herbage must contain

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adequate amounts of soluble carbohydrates which, in the case of grasses, include the free sugars—glucose, fructose and sucrose; the oligosaccharides—stachyose, raffinose, melibiose; and fructosan.[2]

A number of workers[3–5] have noted that, in silage studies, the production of total volatile acids and lactic acid may be greater than the loss in soluble carbohydrates; this suggests that substances other than sugars and fructosans are converted into acids. Whittenbury[6] has pointed out that citric and malic acids in herbage may be dissimilated or used as energy sources by lactic acid bacteria and, since these acids disappear during ensilage,[7] lactic and other organic acids are likely to be the end products of their fermentation.

Possible further sources of lactic and volatile fatty acids are amino-acids obtained from proteolysis during ensilage but, since ammonia is not produced to any extent in well-preserved silage,[8] amino-acids are not likely to play an important rôle in this respect.

A few workers have commented on the disappearance of hemicelluloses during ensilage. Harwood[9] suggested that in the absence of sufficient water-soluble carbohydrates, lactobacilli survive by attacking araban and xylan. Orla-Jensen[10] has stated that pentosans are probably fermented by *Betabacterium pentoaceticum* (*Lactobacillus brevis*) in silage and de Man[11] showed that *Streptobacterium casei* (*Lactobacillus casei*) was able to ferment galactan in potato pulp sterilised previously with ethylene oxide.

In a number of silage experiments carried out by McDonald *et al.*[8, 12] with *L. italicum* and *D. glomerata*, the hemicelluloses of herbage and silages were determined and reported as galactan + araban + xylan. In 20 silage trials, the loss of hemicelluloses during ensilage was 31·1% (S.E. ±2·6). The individual losses of polymer amounted to: galactan 29·2% (S.E. ±6·0), araban 52·7% (S.E. ±3·7) and xylan 24·8% (S.E. ±3·4). The results of these studies indicated that considerable breakdown of hemicellulose occurred during ensilage.

Wylam[13] isolated an enzyme from *L. perenne* which was able to breakdown hemicellulose to pentoses and had an optimum pH value 5–6 although in this work no quantitative measurements were made. There is evidence that pentose sugars are, at least in part, the end products of decomposition of hemicellulose during ensilage since the main residual sugars present in silage extracts are xylose and galactose.[8] Although pentoses are readily available to many lactic acid bacteria there is a lack of knowledge about the extent and cause of hemicellulose breakdown during ensilage.

It is possible that hemicellulose decomposition may occur in three ways: (1) action of hemicellulases present in the original herbage; (2) bacterial action and (3) hydrolysis by organic acids produced during fermentation. The purpose of the present investigation was to study the effects of each of these processes. The activity of hemicellulases was studied over a range of pH and temperature which could occur during the first few days of the ensilage process.

Experimental

Chemical analysis

Total reducing sugars were determined by the titrimetric method of Somogyi.[14] Chromatographic separation of hemicellulose hydrolysates was carried out by the method described by McDonald *et al.*,[8] individual sugars eluted from chromatograms being determined colorimetrically with 0·2% benzidine in acetic acid.[15]

Preparation of hemicellulose

The method of preparation of the hemicellulose was similar to that adopted by Aspinall & Cairncross[16] for *D. glomerata*.

L. perenne was extracted with boiling ethanol–water (4 : 1) to remove soluble sugars and colouring matter, and with boiling water to remove water-soluble polysaccharides. The residue was delignified with acidified chlorite solution.[17] The resulting holocellulose was extracted twice with N-sodium hydroxide solution for 8-h. periods. After clarification by filtration through paper pulp, the extract was acidified to pH 5 with acetic acid and the hemicellulose precipitated by the addition of acetone. The hemicellulose was washed by decantation with graded strengths of acetone and water until the strength of acetone was 95% (v/v), filtered, washed with acetone and ethanol and dried in a vacuum desiccator. The hemicellulose was further purified from acetic acid solution (pH 5) by reprecipitation with acetone, filtered and dried under vacuum.

Composition of hemicellulose

The purified hemicellulose obtained from *L. perenne* was hydrolysed with N-sulphuric acid and, after neutralisation of the solution, the resultant sugars were separated chromatographically and determined colorimetrically by the method previously described. The hemicellulose contained xylose (58·5%), arabinose (14·6%), glucose (6·1%), galactose (5·7%), together with a small amount of ash (1·3%) and unidentified material, some of which may have been uronic anhydride.[18]

Preparation of enzymes

Enzyme preparations were made from freshly cut samples of *L. perenne*, *L. italicum* and *D. glomerata*, all at the young leafy stage of growth. Samples (250 g.) of the chopped grass were extracted with 0·25% sodium carbonate (2 l.) for 8 h. at 4°. The mixture was filtered through muslin and centrifuged. The supernatant liquid was decanted and dialysed against running water for 4 days. Ammonium sulphate was added, with stirring, to 75% saturation to precipitate the enzymes. The mixture was set aside overnight at 4°, the bulk of the supernatant liquid was decanted off and discarded. The remainder was centrifuged, the precipitate dissolved in water and dialysed for 3 days against running water to remove final traces of ammonium sulphate. The dialysed material was centrifuged and the supernatant liquid freeze-dried to give the enzyme preparation (approx. 0·7 g.).

Effect of enzyme preparations on hemicellulose

(a) *Short-term effects*.—The activity of the enzymes extracted from the three grasses was measured by the increase in reducing value produced by each enzyme in hemicellulose solution. The effects of time, temperature and pH value were studied, the experiments being planned according to a statistical design so that interactions, as well as the main effects, could be studied. The design was a $3 \times 3 \times 6$ factorial with 4 replications. The 6 temperature levels were used as blocks. On account of limitations in incubator space the experiment was carried out in two batches, with an interval of 7 days between each batch.

Tubes were made up containing 1 ml. of 1% purified hemicellulose solution, 1 ml. of solution containing 1 mg. of the enzyme preparation, 2 ml. of a phosphate–citric acid buffer solution, 0·25 ml. of chloroform and 0·25 ml. of toluene. Three levels of pH (4, 5 and 6) were used. The tubes were stoppered and randomised in wire baskets for incubation at 22°, 30° and 37°, in the first batch, and at 43°, 50° and 57° in the second batch. Blank determinations were carried out with 1 ml. of water instead of enzyme solution. The reducing value of each solution after centrifuging was examined at intervals of 1, 2 and 3 days.

Preliminary studies had demonstrated that, in the absence of chloroform and toluene, the pentoses produced in the solution were rapidly broken down by bacterial activity. Throughout these experiments bacterial counts were negligible.

(b) *Long-term effects*.—An experiment of a similar design to the first was carried out over periods of 7, 14 and 28 days.

Chemical hydrolysis of hemicellulose

Tubes containing 1 ml. of 1% hemicellulose solution were incubated at 37° for a period of 90 days with buffer solutions having pH values of 4, 5 and 6, for measurement of the breakdown of hemicellulose by acid hydrolysis. Tubes containing known amounts of xylose were also incubated to determine if any destruction of sugar took place.

Media used for detecting bacterial breakdown of hemicellulose

A solution of hemicellulose was sterilised by either Seitz-filter or autoclaving and then added to glucose soft agar or basal soft agar to give a final concentration of 1·0% (w/v). Glucose soft agar consisted of meat extract (Lab Lemco), 0·5% (w/v); glucose, 0·1% (w/v); peptone

(Evans), 0·5% (w/v); Tween 80, 0·05% (v/v); yeast extract (Difco), 0·5% (w/v); agar (Davis), 0·15% (w/v); and tap water. Bromocresol purple was added as a pH indicator and the medium adjusted to pH 6·5 before it was autoclaved at 15 p.s.i. for 15 min. Basal soft agar was similar to the above but did not contain glucose. The complete hemicellulose-containing media were inoculated whilst still liquid at 37–40° with a capillary-pipette drop of turbid culture from a liquid medium similar to glucose soft agar, but lacking agar and containing glucose at 0·5% (w/v).

The cultures used consisted of lactic acid bacteria originally isolated from silage. Their ability to break down hemicellulose was judged by the increase in growth and acidity over that occurring in hemicellulose-free media.

Results

The effects of time, pH and temperature on the action of each of the grass enzymes on the hemicellulose preparation were measured by determining the quantity of reducing sugars produced.

In the short-term experiment, sugar production was always greatest after the longest period of incubation (3 days), the highest amount being produced with the *L. perenne* enzyme at pH 6·0 and a temperature of 37°. The sugar produced under these conditions was 14·8 mg. (as xylose) per 100 mg. of original hemicellulose. The maximum production of sugar with both the *L. italicum* (14·4 mg.) and *D. glomerata* (10·9 mg.) enzymes occurred at the same pH.

The main effects of the three variables studied are summarised in Fig. 1. Enzyme action increased with pH in every case up to a maximum at pH 6. In every case too, the enzyme action increased significantly with time. In considering the main temperature effect, the enzymes from *L. perenne* showed greatest action at 37°, that from *L. italicum* at 30° and that from *D. glomerata* at 43°, but temperature in the range 30–43° did not affect enzyme action as much as pH and time.

The interactions between the three main effects were also examined statistically and the most interesting was that between temperature and pH (Fig. 2). The activity of the two ryegrass enzymes was significantly lower at 37° and pH 4 than at 30° and the same pH, although the optimum temperature of the enzymes from *L. perenne* at pH 5–6 was 37°.

In the experiment designed to study the long-term effects of enzymes on hemicelluloses, it was found that the amounts of reducing sugars produced after 14 and 28 days' incubation were similar to that produced after 7 days, showing that enzyme activity had ceased after a

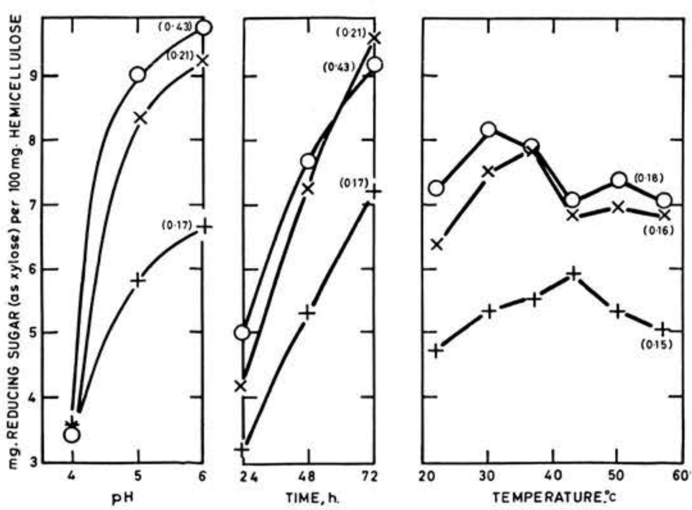

FIG. 1.—*Effects of pH, time and temperature on production of reducing sugars by hemicellulases*
× *L. perenne* enzyme ○ *L. italicum* enzyme + *D. glomerata* enzyme
(numerals in brackets are LSD values)

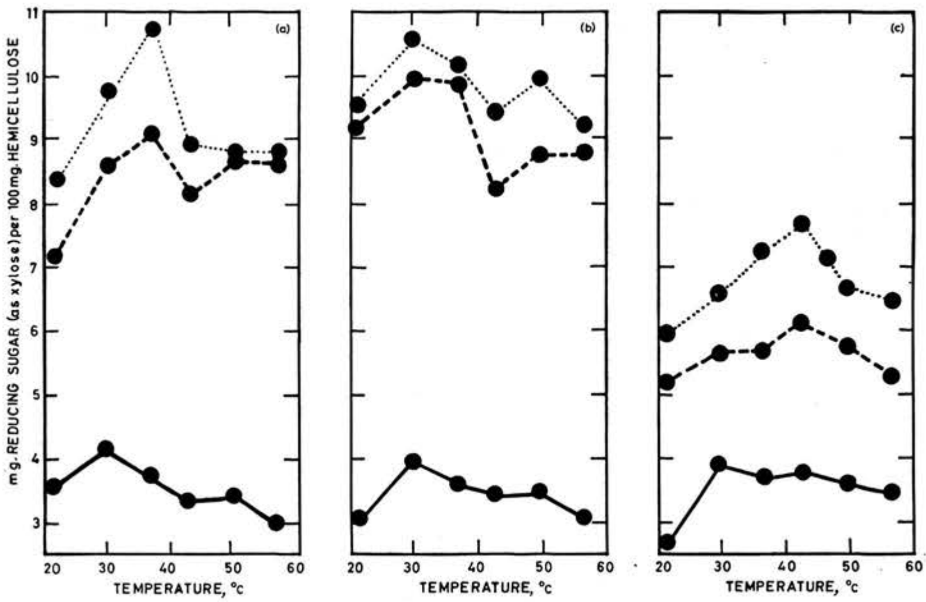

FIG. 2.—*Interaction between pH and temperature on the production of reducing sugars from hemicellulose by different grass enzymes*

(*a*) *L. perenne* enzyme (*b*) *L. italicum* enzyme (*c*) *D. glomerata* enzyme

●———● pH 4 ●- - - - -● pH 5 ●·········● pH 6

7-day period. The results of the 7-day experiment are shown in Table I. The maximum sugar content was produced by the *L. perenne* enzyme at pH 6 and 37°. The proportions of individual sugars produced from the hemicellulose breakdown at pH 6 and 37° are shown in Table II.

The results in Table III show the pH effect on the hemicelluloses over a period of 90 days. The relatively high quantity of sugars produced at pH 4 suggest that considerable breakdown due to chemical hydrolysis may occur in low pH silages stored for a long period of time.

In the bacteriological studies the following organisms were tested for their action on hemicellulose: *Lactobacillus plantarum* (20 strains); *Lb. casei* (15 strains); *Lb. brevis* (30 strains); *Lb. buchneri* (20 strains); *Lb. fermenti* (5 strains); *Leuconostoc mesenteroides* (100 strains). None of these organisms had a detectable effect on the hemicellulose preparation, although all were able to utilise one or other of the pentoses which would result from hemicellulose breakdown.

Discussion

The amount of enzyme action on the hemicellulose preparation was measured by determination of the amount of free sugar and, although this may not give a completely true picture of the breakdown that occurred, it was justifiable in that production of free sugar is important in the ensilage process. This technique of measuring the hemicellulase activity from the amount of reducing sugar produced has been used by Walker & Hopgood.[19]

The three enzymes studied were similar in as much as enzyme action increased with time over a 3-day period. The results showing the effect of pH on enzyme activity agree with those of Walker & Hopgood, who studied the activity of a purified enzyme isolated from sheep rumen microflora on a hemicellulose prepared from wheaten hay chaff. They also found that the optimum pH for maximum hemicellulose breakdown was pH 6.

The different optimum temperatures for the three enzymes—*L. italicum* (30°), *L. perenne* (37°) and *D. glomerata* (43°)—were also surprising. Throughout the experiments the sugar values obtained for *D. glomerata* enzyme were consistently lower than those obtained for the

Table I

Reducing sugars produced after incubation of hemicellulose for 7 days with grass enzymes

(Results as mg. of xylose/100 mg. of hemicellulose)

pH	Enzyme source: *L. perenne* 22°	30°	37°	*L. italicum* 22°	30°	37°	*D. glomerata* 22°	30°	37°
4	12·0	12·0	9·0	9·5	11·5	11·8	11·1	11·6	9·8
5	17·0	17·9	21·6	17·3	18·5	20·3	16·0	16·0	17·4
6	20·8	21·8	22·8	18·5	20·5	20·8	15·8	16·5	17·2

Table II

Relative quantities of reducing sugars (as % total reducing sugars), resulting from the enzymolysis of L. perenne *hemicellulose for 7 days at 37°*

Sugar	Enzyme source: *L. perenne*	*L. italicum*	*D. glomerata*
Xylose	42·6	47·3	60·1
Arabinose	44·0	40·1	26·9
Galactose	6·1	6·1	6·3
Glucose	7·2	6·5	6·7

Table III

Effect of pH on the hydrolysis of hemicellulose over a 90-day period

(Results as mg. of xylose/100 mg. of hemicellulose)

pH	Temperature 22°	30°	37°
4	7·1	7·6	8·4
5	4·6	4·8	4·8
6	3·8	4·4	4·4

two other enzymes. This may have been due in part to the specificity of the *Lolium* spp. enzymes for the hemicellulose originally isolated from *L. perenne.*

In the experiment designed to study the long-term effects of the enzymes, there was no increase in reducing value after 7 days, indicating that enzyme activity was complete at the end of this time.

Since the fermentation reactions which occur during the first few days of the ensilage process are considered to be of most importance in influencing the type of preservation, any liberation of pentoses during the first week after ensiling could form a useful contribution to the fermentable sugars. The maximum sugars produced were 22·8 mg. per 100 mg. of hemicellulose, so that, assuming that herbage contains about 15% of hemicelluloses (dry matter basis), the additional sugar available for fermentation might be about 3%. It is difficult to assess the practical importance of hemicelluloses as a source of fermentable carbohydrate since maximum enzyme action occurs about the pH 6 level. At lower pH levels enzyme activity is considerably reduced and the production of pentoses may then result more slowly, but over a much longer period, from acid hydrolysis. As the enzyme activity in the temperature range examined did not vary greatly, it is unlikely that the amount of sugar produced by enzyme action will be influenced to any marked extent by temperature variation over the normal range during ensilage. Because of the many variables it is perhaps more expedient for practical purposes to assess the 'available carbohydrates' in terms of soluble sugars and fructosan as is the usual practice.

It is interesting to compare the products of hydrolysis after enzymolysis for 7 days with the results of hydrolysis of the original hemicellulose. The arabinose/xylose ratio in the latter is approximately 1 : 4, whereas the ratio in the products of enzymolysis is approximately 1 : 1 for the two *Lolium* spp. enzymes and about 1 : 2 for the *D. glomerata* enzyme. The increase in this ratio indicates that arabinose has been split preferentially from the xylan chain by the enzyme. This finding is in keeping with the results obtained by McDonald *et al.*[8] for silages. Aspinall & Cairncross[16] studied the effect of mild acid hydrolysis of hemicellulose extracted from *D. glomerata* and showed that arabinose was preferentially removed. It is possible that the relatively high quantities of arabinose produced during enzymolysis in the present studies may have been due to the greater accessibility of the arabinose residues because of their position as side chains to the main xylan chain. An alternative although unlikely explanation is that the enzyme preparation contained a specifically arabinose-splitting enzyme.

In the bacteriological studies only lactobacilli and leuconostocs were examined. These lactic acid bacteria were considered to be the only organisms likely to survive the depletion of soluble sugars in a well-made silage. None of the species tested gave any indication of being able to break down hemicellulose. Our results therefore do not support the hypothesis of Harwood[9] that lactobacilli attack hemicelluloses or the suggestion of Orla-Jensen[10] that *Betabacterium pentoaceticum* (*Lactobacillus brevis*) probably ferments pentosan.

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THE EFFECT OF HISTAMINE ON SILAGE DRY-MATTER INTAKE

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The oral introduction of 0·5 g histamine as the diphosphate into the rumen of sheep produced no effect on the physiological condition of the animals. The feeding of histamine, as the dihydrochloride, in levels up to 1 g/day to sheep on silage diets did not affect the dry-matter intake.

INTRODUCTION

It has been shown that the dry-matter intake of silage by cows is frequently lower than the intake of hay dry matter (10). Even although consumption of silage dry matter is positively related to silage dry-matter content (8, 10), the decrease in intake is not due to the water in the silage *per se* but to some changes in fermentation associated with wet herbage (7). Moore *et al.* (7) postulated that some constituent formed during the fermentation process, possibly in the nitrogen fraction, affected the appetite of the animal.

In a recent paper Macpherson (6) confirmed the presence of histamine in grass silage. In six silages examined, the histamine content of the dry matter ranged from a trace to 844 mg/kg.

Dain *et al.* (1) have reported that intravenous injection of histamine into a sheep completely stopped rumen motility and the ability of the animal to eructate. These workers also demonstrated the presence of histamine in rumen ingesta of sheep on a high cereal diet, and found that if the concentration in the rumen ingesta was 20 μg/ml the animals were acutely ill but recovered; at concentrations greater than 70 μg/ml, however, the animals died. It was also reported that, normally, sheep ingesta were practically free of histamine.

The presence of histamine in silage and its potential toxic action on the animal suggested that this amine might be an important fermentation product causing low silage dry-matter intakes. The following experiments were designed to study the effect of oral administration of histamine to wether sheep.

EXPERIMENTAL AND RESULTS

Absorption of histamine

In a preliminary experiment to study the clinical effects, if any, from the oral administration of histamine, 4 Cheviot wether sheep of similar age (15 months) and size (45 kg) were divided into two groups. Each animal in group A was dosed orally with a gelatine capsule containing 0·5 g histamine as the diphosphate. This dose was determined on the basis of the potential intake of histamine by a sheep eating histamine-containing silage, and the method of administration was based on the observation that solid matter found its way to the rumen rather than the abomasum (2).

The two animals in the control group B were given a capsule containing 0·5 g of glucose instead of histamine. Before the capsule was administered, blood samples were removed from the jugular vein. For three weeks preceding and during the trial, the four sheep were fed fresh grass (predominantly *Lolium perenne*).

Rumen movements, respiration and pulse rates were recorded before treatment and at hourly intervals after administration of the capsules. During the experiment none of the above systems recorded deviation from the normal. Histamine was not detected in blood samples taken at hourly intervals for 4 hours after dosing.

Effect of added histamine on silage intake

In the clinical trial described above, the histamine was given in the form of a capsule to sheep on a grass diet. In order to study any possible effect of the amine on silage intake it

TABLE 1. Composition of silage fed during intake trial
(per cent dry matter)

*Dry matter	23·06	Lactic acid	14·62
Organic matter	90·76	Formic acid	0·18
Crude protein	12·96	Acetic acid	2·92
Ether extract	3·22	Butyric acid	Nil
Crude fibre	26·02	Histamine	trace
Nitrogen-free extractives	48·56		
Cellulose	29·11		

*Corrected for volatile losses

was decided to simulate the normal dietary pathway of the histamine by using a solution and mixing it intimately with the silage.

The silage, made from mixed herbage species (predominantly *Lolium perenne*), was fed to two groups of Cheviot wether sheep (age 15 months, weight 49 kg) in a cross-over trial. To one group was fed 1 g of histamine as the dihydrochloride dissolved in 10 ml distilled water. In the first part of the trial this histamine solution was sprayed over a portion (about $\frac{1}{10}$) of the silage ration and fed first before introducing the non-treated silage into the feed box. This histamine-treated silage was normally consumed within 45 minutes. A similar procedure was adopted with the control group, using 10 ml distilled water in place of histamine solution.

In the second part of the cross-over trial the histamine solution or water was sprayed over the whole daily ration allocated to each animal. Although this second method ensured a more even intake of histamine, only about 0·6 g histamine was consumed daily by each animal because of the relatively high feed residues.

Each treatment lasted 7 days with a preliminary feeding period of 7 days and a recovery period of 7 days between treatment changes. Feed residues were collected and weighed daily. In all cases daily silage residues were never less than 15% of the daily ration consumed. Owing to the shortage of silage the final recovery period (5th) was restricted to 5 days.

During the trial the sheep were housed in individual crates normally used for digestibility studies (5).

The composition of the silage fed is given in Table 1. The silage was well preserved, of low pH value (3·6) and contained only traces of histamine. The daily intakes of dry matter for the two groups of animals are shown in Fig. 1 and the summarized results are given in Table 2. The overall mean intake of silage dry matter on the histamine-fed groups (856 g/day) was slightly higher than the intake of the corresponding control group (816 g/day) but these differences are obviously of no significance.

DISCUSSION

Histamine is thought to be produced in silage by the action of specific bacterial decarboxylases on histidine liberated in the extensive proteolysis associated with silage fermentation (6). The exact conditions under which histamine is produced are uncertain, but it is not necessarily present in all silages.

Although it is known that the minimal lethal doses of histamine given intravenously to various animals are extremely low (3), histamine is not well absorbed from the gastro-intestinal tract (9). The potential toxic effect of histamine present in silages will depend not only upon the concentration but upon the absorption of the amine from the tract. That absorption from the tract can occur has been established with sheep and cows fed rations

TABLE 2. Dry matter intakes of silage

	Group A		Group B	
Period	Treatment	Mean dry-matter intake g/day	Treatment	Mean dry-matter intake g/day
1.	Silage	998	Silage	897
2.	Silage + histamine	904	Silage	816
3.	Silage	920	Silage	833
4.	Silage	815	Silage + histamine	807
5.	Silage	944	Silage	927

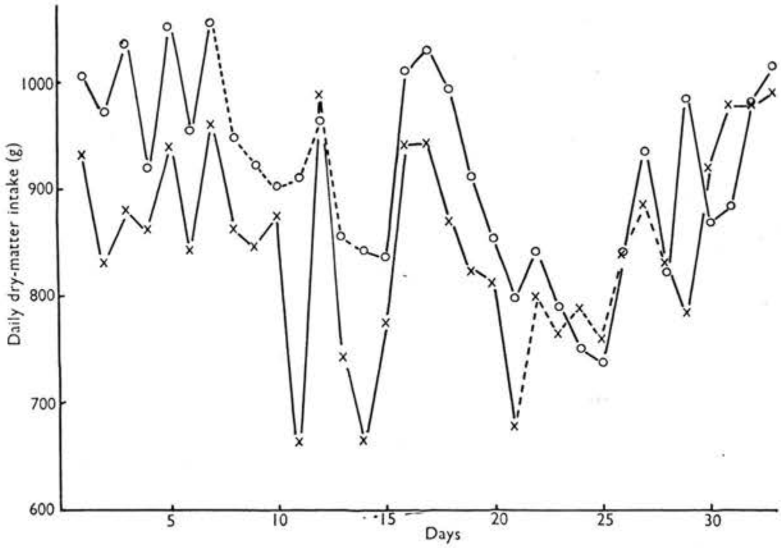

Fig. 1. Silage dry-matter intakes of control and histamine-fed sheep.

○ = Group 1 × = Group 2 ----- = period of histamine administration.

high in starch (1, 4). The conditions under which histamine is absorbed from the rumen are not fully understood, but there appears to be a correlation between ruminal hydrogen-ion concentration, histamine production and illness of the animal (1).

In our studies the introduction of 0·5 g histamine as the diphosphate into the rumen of sheep produced no effect on the physiological condition or well-being of the animal.

From an examination of the venous blood, sampled at regular intervals over a four-hour period after capsule administration, there was no evidence of histamine absorption. When quantities of histamine up to 1 g were given orally as the dihydrochloride mixed with grass silage over a 7 day period, dry-matter intake was not reduced. Our results with silage agree with those of Shinozaki (9), who found that the oral administration of 1–3 g of histamine as the dihydrochloride had no effect on adult sheep.

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THE POTENTIAL VALUE OF SILAGE EFFLUENT AS A FERTILIZER

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Results of the analysis for dry-matter, nitrogen and major base content of 9 silage effluents obtained from farm silos and 3 effluents from experimental tower silos are presented. The mean values obtained for the principal plant nutrients in the 12 effluents examined were 0·19% N, 0·037% P and 0·38% K. The results indicate that effluents may be a useful source of plant nutrients, particularly when undiluted with rainwater, and that, in general, silage effluents appear to be superior to liquid manure in manurial value.

INTRODUCTION

There is experimental evidence (4) to suggest that the volume of effluent produced during ensilage tends to be inversely related to the dry-matter content of the crop ensiled, although the degree of consolidation and the condition of the crop are other important factors controlling effluent production (3). It follows that, where wet or succulent crops have to be ensiled, there are increased risks of producing large amounts of effluent and, in addition, the silage produced is more likely to be badly preserved. It is therefore desirable to ensile material relatively high in dry matter, and normally a content of more than 25% is recommended. With most forage crops this involves a period of wilting prior to ensilage and this may not always be practicable. On the farm large volumes of silage effluent may be produced from time to time, and the problem of disposing of this material may be a serious one.

Because of their acidic reaction and relatively high content of soluble carbohydrate, most silage effluents are favourable media for the growth of fungi and actinomyces, and the presence of effluent in drains or streams is therefore very undesirable. In addition, the disposal of effluents in this way represents an irretrievable loss of plant nutrients from the land, inconsistent with sound agronomic practice. In Scotland the pollution of streams and rivers has been an offence in law since 1951.

It is, perhaps, not generally realized that a silage effluent may contain a substantial proportion of the N and K originally present in the crop ensiled (5), so that the economic loss can be considerable where nitrogenous and potassium fertilizers are regularly used. On some farms it is now the practice to drain the effluents into tanks which are also used for the storage of liquid manure, and this not only solves the problem of disposal but provides a means of reducing the losses of N, K and other soluble plant foods. Another possibility which has been suggested by Moore and Walker (4) is that the effluent should be directly applied as a top dressing to grass or root crops at a rate of 10–12 tons per acre.

With a view to determining the possible manurial value of silage effluents the authors have carried out analyses of effluents obtained from 9 different farm silos and 3 experimental silos and have compared their nitrogen and major mineral contents with those of samples of liquid manure (gülle).

MATERIAL AND METHODS

The farm effluents were obtained from drainage sumps sited near the silos, and, so far as could be ascertained, the sumps were not used for drainage other than from the silos. However, in some cases, the possibility of dilution with rainwater could not be ruled out. All the silos had been covered either with a roof or with polythene sheeting and were of the surface-clamp type. With the exception of

TABLE 1. Composition of silage effluents (g/100 ml)

Farm samples	Dry matter	Ash	N	P	K	Ca	Mg
1	3·68	0·95	0·183	0·034	0·33	0·077	0·020
2	2·26	0·82	0·102	0·025	0·25	0·070	0·026
3	0·61	0·22	0·039	0·006	0·08	0·020	0·005
4	1·91	0·71	0·123	0·031	0·20	[illegible]	0·010
5	7·50	2·03	0·353	0·070	0·57	0·215	0·057
6	6·82	1·80	0·233	0·032	0·41	0·230	0·033
7	0·66	0·27	0·030	0·008	0·09	0·030	0·005
8	8·37	2·15	0·370	0·072	0·85	0·110	0·036
9	4·54	1·92	0·100	0·012	0·35	0·360	0·038
Mean	4·04	1·21	0·170	0·032	0·35	0·125	0·026
% dry matter	—	29·90	2·970	0·790	8·61	3·090	0·640
Experimental silo samples							
10	4·29	0·98	0·163	0·033	0·23	0·180	0·043
11	6·40	1·37	0·247	0·052	0·61	0·086	0·027
12	8·74	1·91	0·327	0·075	0·57	0·240	0·083
Mean	6·48	1·42	0·246	0·053	0·47	0·169	0·051
% dry matter	—	21·91	3·800	0·820	7·25	2·610	0·790
Liquid-manure samples							
Mean (5 samples)	1·75	—	0·130	0·019	0·13	—	—
% dry matter	—	—	7·420	1·090	7·42	—	—

one sample (No. 6, Table 1) which was obtained from a cereal/legume mixture, all the samples were derived from grass or grass/clover mixtures. All the material ensiled was cut with the forage harvester in the period June to August 1962. The samples were taken in September and October 1962, and at the time of sampling effluent flow had virtually ceased.

Three effluent samples were obtained from small experimental tower silos of 1000 kg capacity in which either *L. multiflorum* or *D. glomerata* was ensiled. Details of these silos and methods of filling and effluent collection have been described elsewhere by McDonald *et al.* (3).

For comparative purposes, 5 samples of liquid manure were examined. These samples were obtained from sumps draining from open yards or byres housing fattening cattle or dairy cows.

In all the analyses, N was determined by the Kjeldahl method and K, Ca and Mg by means of a Unicam SP900 flame spectrophotometer. P was determined by quinolinium phosphomolybdate precipitation.

RESULTS

The analytical results obtained for the 12 effluents examined and the mean contents of N, P and K in the 5 samples of liquid manure are given in Table 1.

Dry matter

The dry-matter content of the 9 farm effluents ranged from 0·61 to 8·37 g/100 ml with a mean value of 4·04 g/100 ml. It is clear that the dry-matter content of the effluents is an important factor governing their nutritional value for plants. For example, Sample No. 8, which has the highest dry-matter content among the farm effluents, has also the highest content of N, P and K.

It is accepted that the dry matter of the crop ensiled is an important factor governing the dry matter of the effluent produced (3, 6) and this is confirmed in the effluent samples Nos. 10, 11 and 12 (Table 1), which were produced from grass with dry-matter contents of 14·2, 17·0 and 22·5%, respectively.

In a study, independent of the present investigation, of 190 samples of effluent obtained from small experimental tower silos of the type previously described, the dry matter of the effluent ranged from 2·6 to 14·5 g/100 ml, with a mean value of 6·6 g/100 ml, the lowest value being obtained from herbage with a dry-matter content of only 14·9%. Since there was no possibility of dilution with rainwater in the case of the experimental silos, it seems reasonable to suggest that the abnormally low contents of dry matter in farm effluents Nos. 3, 4 and 7 were caused by such dilution.

The dry-matter contents of the 5 liquid-manure samples ranged from 1·23 to 2·27 g/100 ml, with a mean value of 1·75 g/100 ml. The farm silage effluents were, on the whole, more than twice as concentrated as the liquid manure samples, despite the possibility of dilution with rainwater.

Nitrogen

A mean content of 0·17 g/100 ml was obtained for the farm effluents as compared with 0·13 g/100 ml, with a range of 0·09 to 0·17 g/100 ml, for the gülle samples. The 190 effluents obtained from experimental silos had a mean N content of 0·30 g/100 ml with a range of 0·08 to 0·93 g/100 ml. However, in the latter case, the herbage ensiled included a number of samples containing 18–20% crude protein—a higher content than is generally found in ensiled farm crops—and this has, no doubt, had an effect on the N content of the resulting effluents.

Although the farm effluents compare very favourably with the liquid manure samples examined with regard to N content, the liquid manures are evidently richer in N on a dry-matter basis.

Minerals

The mean contents of K and P for the farm effluents were 0·35 g K/100 ml and 0·032 g P/100 ml, significantly higher values than for the liquid manures which contained 0·13 g K/100 ml with a range of 0·08 to 0·23 g K/100 ml and 0·019 g P/100 ml with a range of 0·012 to 0·22 g/100 ml.

Examination of the K, Ca and Mg contents in Table 1 indicates that the contents of these elements are roughly in the same proportions in which they occur in herbage. As one might expect, on a dry-matter basis, the effluent samples are much richer than herbage in all the mineral elements, and it is clear that, where large volumes of effluent are produced, the loss of minerals represents a substantial fraction of the mineral content of the crop ensiled.

DISCUSSION

Jensen (2) has examined the N, P and K contents of silage effluents and quotes mean values of 0·15%, 0·036% and 0·42%, respectively. This worker has also stated that the availability of the N as a plant nutrient is comparable with that of farm-yard manure. Godden (1) has also determined N, P and K contents of effluents from 3 silage mixtures and gives figures of 0·28%, 0·053% and 0·105%. In general, these values agree quite well with the results presented in the present paper, although the K figure quoted by Godden seems to be abnormally low.

The results of the present investigation indicate that silage effluents are generally similar to samples of liquid manure as regards their content of N and that they are, on the whole, much richer sources of K and P. The P contents of both effluents and liquid manures are, however, rather low. When there is no dilution with rainwater, the differences between the contents of plant nutrients in effluents and liquid manures are considerable, and potentially the effluents can be considered to be much superior as manurial material.

Since the K, Ca and Mg in effluents are present in about the same proportions as in herbage, an effluent used as a fertilizer will provide a supply of the essential major bases in similar proportions to those taken up by plants.

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FERMENTATION STUDIES ON INOCULATED HERBAGES

By P. McDONALD, A. C. STIRLING, A. R. HENDERSON and R. WHITTENBURY

The fermentation changes in herbage inoculated with selected strains of lactobacilli were studied in two experiments. In the first, *Lolium multiflorum* containing 16·2% of soluble carbohydrates was used in four silos, the treatments being: control, inoculated, molassed, inoculated–molassed. All silages were well preserved (pH 3·9–4·1), the gaseous losses being the lowest in the molassed herbages.

In the second experiment, *Dactylis glomerata* of low soluble carbohydrate content (4·3%) was used and the treatments were: control, inoculated, bruised, inoculated–bruised. The material which had been inoculated and bruised was well preserved (pH 4·2) while the non-bruised herbages were badly preserved (pH 4·9) and had higher gaseous losses. The bruised silage without inoculation had pH 4·5.

The results suggest that there may be no advantage in inoculating herbage rich in soluble carbohydrates, but that herbage of low sugar content may benefit from such treatment.

Introduction

In silage making, it is generally assumed that herbage is adequately supplied with the lactic acid bacteria which ferment the soluble carbohydrates of plants. Recent studies, however, have shown that the number of these bacteria on fresh herbage may sometimes be extremely low.[1–3] Nevertheless, if conditions in the silo are suitable for the development of the organisms, sufficient acid may be produced to preserve the silage at a pH level within the range 3·8 to 4·2. There is some evidence, however, that the longer the period of development before a satisfactory low pH is attained, the higher will be the loss of dry matter due to the activity of plant and bacterial enzymes. Various workers have added lactic acid bacteria to herbage in an attempt to secure rapid acidification but a beneficial effect has not always been noted.[4]

The purpose of the present investigation was to study the effect of an inoculum of selected strains of lactobacilli on the chemical and bacterial changes occurring in grass ensiled in small tower silos.

Experimental

Procedure

The silo unit used in this investigation has been described in detail in an earlier publication[5] and consists of four metal silos, each having a maximum capacity of 1000 kg. of fresh herbage, and each being suspended from a weighing device which enables direct measurements of weight changes to be recorded. Preliminary tests showed that the four silos, filled under identical conditions with *Lolium multiflorum* (Italian ryegrass), yielded similar results.[6] In the experiments reported in this paper conditions of filling and consolidation were similar to those described in earlier publications.[6, 7] Consolidation was achieved by covering the ensiled herbage with polythene sheeting, wooden compression discs and stone blocks corresponding to a surface pressure of 37 g./sq. cm.

Temperature measurements were recorded from thermocouples (nine per silo) buried at different levels throughout the ensiled herbage. Effluents were collected daily, or when they appeared, and analysed for dry matter, sugars, total nitrogen and ash. Techniques of sampling, filling, methods of analysis, bacteriological and digestibility methods have been described elsewhere.[6] Dry-matter corrections were made for losses during drying by an oven distillation technique.[8]

Two experiments were carried out. In the first, *Lolium multiflorum* (S22) was cut with a mower on 14th October and ensiled the same day. The treatments were as follows:

Silo A, grass (910 kg.) + water (9·2 kg.)
Silo B, grass (910 kg.) + inoculum (9·2 kg.)
Silo C, grass (910 kg.) + water (9·2 kg.) + molasses (18·2 kg.)
Silo D, grass (910 kg.) + inoculum (9·2 kg.) + molasses (18·2 kg.)

The molasses, which contained dry matter 70·1%, soluble sugars 45·6%, ash 8·6% and nitrogen 0·3%, was diluted with half its weight of water and was sprayed on to the grass from a pressure spray during filling.

The inoculum consisted of a mixture of eight strains of homofermentative lactobacilli selected for their ability to ferment the soluble carbohydrates of grass and for their ability to grow over a range of temperature from 10° to 45°. They were grown in a medium containing molasses; 2 l. of culture were added either to water or to diluted molasses for application by pressure spray to the herbage in silos B and D during filling. Silos A and C were filled and covered first so that there was no possibility of cross-inoculation from the other silos.

The silos were opened 58 days after being filled. The contents were sampled for analysis and digestibility studies with sheep were carried out in triplicate.

The same herbage was used to fill silos in the laboratory to provide material for an investigation of the bacterial changes during the early stages of the fermentation. A count of viable organisms was obtained by plating macerated material (50 g. of grass or silage made up to 300 g. with sterile water) on glucose yeast agar. The numbers of lactobacilli were estimated on acetate agar, those of Gram-negative bacteria on lactate agar and the number of anaerobes by inoculation into a medium containing lactate, acetate and gelatin which indicated the presence of both lactate-fermenting and proteolytic types.

The grass used in the second experiment was *Dactylis glomerata* (cocksfoot) consisting of a mixture of two varieties—Danish and S143. Two different harvesting methods were used; one half of the crop was cut with a mower and ensiled without further mechanical treatment, while the remainder of the crop was cut with a flail-type forage harvester, which caused the grass to be bruised. The treatments were as follows:

Silo A, long grass	(910 kg.)	+ water	(0·5 kg.)
Silo B, long grass	(910 kg.)	+ inoculum	(0·5 kg.)
Silo C, bruised grass	(910 kg.)	+ water	(0·5 kg.)
Silo D, bruised grass	(910 kg.)	+ inoculum	(0·5 kg.)

The inoculum consisted of the strains of lactic acid bacteria used in the first experiment. Only 500 ml. of culture were added. The grass was cut and ensiled on 14th September and the silos opened 150 days later.

Results

Experiment 1

Volume changes

The initial volume of the grass in the silos was 3·02 cu. m. It fell to about half this value in silos C and D by the 6th day, in silo B by the 8th day, and in silo A by the 11th day. The volume occupied by the silage in A remained consistently higher throughout the ensiling period than that in silos B, C and D. At the end of the experiment, on the 58th day, the final volumes were A, 1·22; B, 1·15; C, 1·18; and D, 1·15 cu. m.

Composition

The chemical composition of grass and silages is given in Table I. The grass had a low content of dry matter (15·6%) and contained appreciable amounts of soluble carbohydrates (16·2%). The four silages were well preserved (pH 3·9–4·1) and of relatively high lactic acid content with only traces of butyric acid present.

The pH values of the effluent (Fig. 1) showed minor day-to-day differences between treatments but all followed a similar pattern. On the 6th day after ensiling, the pH value for effluents A, B and C was 5·0 while the value for D had fallen to 4·5. By the 14th day the values were 4·3 for A and C, 4·1 for B and 4·0 for D. Thereafter the values remained fairly consistent at 3·9–4·2.

Temperature changes

The temperature in all four silos remained relatively low, the maximum values reaching 21° (A), 15° (B) and 14° (C and D) within the first week. By the 12th day all values were 14° or below.

Table I

Composition of grass and silages

(% of true dry matter)

	Experiment 1					Experiment 2					
	Grass	Silages				Grass		Silages			
		A	B	C	D	Long	Bruised	A	B	C	D
Dry matter[a]	15·64	15·09	15·51	16·64	16·66	14·22	13·85	14·56	14·98	16·30	16·32
Organic matter	87·7	85·8	86·5	82·7	86·5	90·9	87·3	88·6	87·0	84·2	84·1
Crude protein	13·8	15·2	15·3	13·2	14·0	26·6	27·0	28·0	27·6	26·1	26·0
Ether extract	3·5	4·7	4·6	4·5	4·6	4·7	4·0	5·4	5·4	5·5	5·9
Crude fibre	22·1	23·8	23·8	21·7	22·2	24·2	22·7	25·6	26·1	23·1	22·1
N.F.E.[b]	48·3	42·1	42·8	43·3	45·7	35·5	33·6	29·5	27·9	29·6	30·1
Total N	2·21	2·43	2·44	2·11	2·25	4·25	4·32	4·48	4·41	4·18	4·16
Protein N	1·95	1·20	1·20	1·00	1·15	3·56	3·51	2·09	1·83	2·19	2·23
Non-protein N	0·26	1·23	1·24	1·11	1·10	0·69	0·81	2·39	2·58	1·99	1·93
Volatile N	—	0·19	0·17	0·17	0·12	—	—	0·51	0·58	0·40	0·32
Total sugars	10·2	1·6	1·8	4·5	5·2	3·9	3·8	0·2	0·1	0·3	0·5
Sucrose	2·4	—	—	—	—	—	—	—	—	—	—
Glucose	2·3	—	—	—	—	—	—	—	—	—	—
Fructose	3·8	—	—	—	—	—	—	—	—	—	—
Oligosaccharides[c]	1·7	—	—	—	—	—	—	—	—	—	—
Fructosan	6·0	0·3	0·2	0·4	0·5	0·4	0·5	0·03	0·02	0·03	0·03
Cellulose	25·1	28·5	28·4	25·3	26·6	27·2	26·2	29·5	29·2	26·8	26·2
Lignin	6·5	6·3	6·3	6·1	6·0	9·2	10·2	8·1	8·1	8·7	8·6
Lactic acid	—	11·5	11·7	9·3	10·5	—	—	3·7	3·3	5·8	7·4
Formic acid	—	nil	nil	nil	nil	—	—	nil	0·2	nil	nil
Acetic acid	—	1·8	1·6	1·5	1·4	—	—	5·8	5·9	4·7	3·5
Propionic acid	—	nil	nil	nil	nil	—	—	nil	nil	nil	0·2
Butyric acid	—	0·2	0·2	0·2	0·2	—	—	0·7	0·5	0·5	nil
pH[a]	6·3	4·1	3·9	4·0	3·9	6·4	6·4	4·9	4·9	4·5	4·2

[a] pH and dry matter determined on fresh material
[b] N.F.E. = nitrogen-free extractives [c] excluding sucrose

Losses

The total weights of silage removed from the silos were A, 772; B, 750; C, 773; and D, 764 kg. Surface waste was low, amounting to A, 1·4; B, 4·0; C, 1·2; and D, 2·5% of the total silage removed. The total effluent weights obtained were A, 138; B, 160; C, 137; and D, 138 kg.

The losses of dry matter and its components are shown in Table II. The gaseous dry-matter losses were A, 13·0; B, 12·6; C, 5·3; and D, 9·4%. The gaseous nitrogen loss was small in all silos and did not exceed 3%.

Small losses of cellulose[9] occurred (2·2–6·6%); this material however cannot be regarded as pure glucan as it contains some pentosan material.[6]

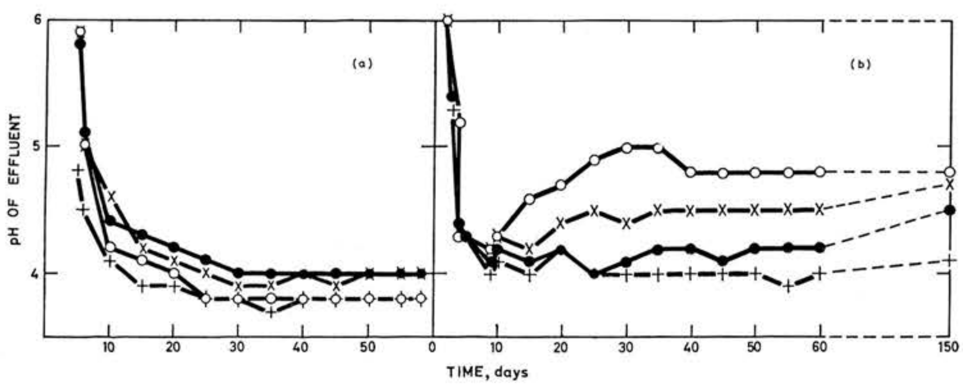

FIG. 1.—*pH values of effluent after various times*

(*a*) Experiment 1 (*b*) Experiment 2

× = Treatment A ○ = Treatment B ● = Treatment C + = Treatment D

Table II

Percentage losses during ensilage

	Experiment 1								Experiment 2							
	A		B		C		D		A		B		C		D	
	Total	Effluent	Total	Effluent	Total	Effluent	Total	Effluent	Total	Effluent	Total	Effluent	Total	Effluent	Total	Effluent
Total fresh	15·2	14·1	17·6	16·7	17·7	14·0	16·1	15·2	12·5	11·4	16·9	15·8	22·6	21·6	23·2	22·2
Dry matter	17·9	4·4	18·9	4·9	14·1	6·2	14·1	6·2	10·2	3·0	12·5	3·7	9·0	6·9	9·7	8·2
Crude protein	9·4	6·8	9·9	7·1	10·0	6·5	6·2	6·5	5·2	4·0	9·2	5·3	12·0	9·4	13·1	11·7
Ether extract	+9·0	—	+5·4	—	+19·2	—	+21·9	—	+4·7	—	+2·0	—	+24·5	—	+32·9	—
Crude fibre	11·6	—	12·7	—	7·8	—	5·7	—	4·9	—	5·4	—	7·4	—	11·9	—
N.F.E.*	28·3	—	28·1	—	27·5	—	23·6	—	25·3	—	31·3	—	20·0	—	19·1	—
Total N	9·4	6·8	9·9	7·1	10·0	6·5	5·8	6·5	5·2	4·0	9·2	5·3	12·0	9·4	13·1	11·7
Protein N	49·5	—	50·1	—	51·9	—	47·1	—	47·3	—	55·1	—	43·7	—	42·1	—
Sugars	87·1	5·3	85·6	6·8	73·9	14·8	69·8	14·2	95·8	2·6	97·1	2·3	93·2	10·8	88·6	13·6
Fructosan	95·8	—	97·3	—	93·7	—	92·1	—	93·5	—	95·4	—	95·1	—	95·3	—
Cellulose	6·6	—	8·1	—	5·2	—	0·4	—	2·4	—	5·9	—	7·0	—	9·7	—
Lignin	20·7	—	21·7	—	12·1	—	13·7	—	22·1	—	22·7	—	22·1	—	23·3	—
Water	14·7	15·9	17·4	19·3	13·6	16·7	16·5	17·0	12·6	12·8	17·6	17·8	24·7	24·0	25·5	24·5

* see Table I

Nutritive value

The digestibility coefficients and digestible nutrients for the grass and silages are shown in Table III. The digestibility values of the grass and silage organic matter were similar.

Table III

Digestibility (D) (%) and digestible nutrients (DN) (%)

	Grass				Silages							
					A		B		C		D	
			D	DN	D	DN	D	DN	D	DN	D	DN
Experiment 1												
Organic matter			76·8	67·4	76·5	65·6	76·9	66·5	76·0	62·9	76·4	66·1
Crude protein			66·2	9·1	68·7	10·4	68·8	10·5	65·3	8·6	66·5	9·3
Ether extract			52·5	1·8	56·3	2·6	60·1	2·8	59·7	2·7	60·0	2·8
Crude fibre			82·5	18·2	85·0	20·2	84·5	20·1	84·2	18·3	84·5	18·8
N.F.E. [a]			78·9	38·1	76·4	32·2	77·2	33·0	76·6	33·2	76·8	35·1
S.E. [b]			—	61·9	—	60·2	—	61·4	—	58·4	—	61·5
T.D.N. [c]			—	69·5	—	68·7	—	69·9	—	66·2	—	69·5
	Long		Bruised									
Experiment 2	D	DN	D	DN								
Organic matter	75·4	68·5	74·8	65·3	83·7	74·2	82·8	72·0	80·9	68·0	78·5	66·1
Crude protein	79·0	21·0	77·8	21·0	88·2	24·7	87·6	24·2	85·2	22·2	83·7	21·8
Ether extract	49·2	2·3	45·4	1·8	77·5	4·2	74·4	4·0	74·8	4·4	74·6	4·1
Crude fibre	81·8	19·8	82·9	18·8	88·5	22·7	87·6	22·9	86·3	19·1	85·1	19·7
N.F.E.	71·6	25·4	70·6	23·7	76·8	22·7	75·0	20·9	73·9	22·3	71·3	21·1
S.E.	—	62·3	—	59·2	—	69·2	—	66·5	—	64·3	—	66·5
T.D.N.	—	71·3	—	67·6	—	79·6	—	77·9	—	73·5	—	71·8

[a] see Table I
[b] S.E. = starch equivalent
[c] T.D.N. = total digestible nutrients

Bacteriological studies

The number of lactic acid bacteria on the fresh herbage was relatively small; lactobacilli and streptococci were present in approximately equal numbers and amounted to 2000/g. dry wt. of grass. This number was raised to 34 million/g. by the addition of the inoculum. Bacterial counts and pH values are given in Table IV. The Gram-negative bacteria numbered 2 million/g. dry wt. on the fresh herbage. By the 3rd day their number had increased to 5 million in the untreated and molassed laboratory-made silages but had decreased to a negligible value in the inoculated silages in which the pH had fallen below 4. By the 9th day they were not detected in any 'tube' silo. After 3 days both streptococci and pediococci had developed in the untreated and molassed silages and heterofermentative lactobacilli were isolated from the molassed material after 9 days. At all the examinations, homofermentative lactobacilli were the dominant organisms in the inoculated silages.

When the large silos were opened after 58 days, the bacteria isolated from the untreated

Table IV

pH values and bacterial counts from grass and silage

Experiment 1

		Bacterial count (millions/g. dry wt.) on glucose yeast agar	Bacterial count (millions/g. dry wt.) on acetate agar	pH
Fresh grass				
untreated		530	<0·01	6·3
+ molasses		900	<0·01	
+ culture		750	34	
Laboratory-made silage at 30°				
Untreated	3 day	3300	1700	5·2
	9 ,,	930	1000	4·7
	142 ,,	28	28	4·4
+ culture	3 ,,	15,000	20,000	3·9
	9 ,,	2900	2900	3·7
	142 ,,	38	28	3·7
+ molasses	3 ,,	2600	2300	4·6
	9 ,,	1600	2800	4·3
	142 ,,	74	126	4·2
+ molasses and culture	3 ,,	17,000	20,000	3·7
	9 ,,	2700	2900	3·7
	142 ,,	20	12	3·7
Large silos				
A untreated		920	950	4·1
B + culture		1400	2000	3·9
C + molasses		611	1100	4·0
D + molasses + culture		2100	2000	3·9

Experiment 2

		Bacterial count (millions/g. dry wt.) on glucose yeast agar	Bacterial count (millions/g. dry wt.) on acetate agar	pH
Fresh grass		107	<0·01	6·4
+ culture		107	6	
bruised		770	0·2	
bruised + culture		1200	12	
Laboratory-made silage at 30°				
Untreated	3 day	1200	3200	4·5
	8 ,,	9300	3800	5·2
	160 ,,	42	42	5·9
+ culture	3 ,,	8500	6000	4·2
	8 ,,	2900	1300	4·5
	160 ,,	42	51	4·5
bruised	3 ,,	1500	3300	4·4
	8 ,,	5100	7200	4·4
	160 ,,	17	17	5·7
bruised + culture	3 ,,	6500	14,000	4·5
	8 ,,	9700	7200	4·4
	160 ,,	13	4	5·5
Large silos				
A untreated		1500	4700	4·9
B + culture		4200	4700	4·9
C bruised		1200	3200	4·5
D bruised + culture		1500	1500	4·2

and the molassed silages comprised both pediococci and lactobacilli. Cocci were not detected in the inoculated silages; in these, lactobacilli were dominant. Anaerobes were present in all four silages. They were most numerous in the molassed material and fewest in the silo to which bacterial culture alone had been added.

Experiment 2

Volume changes

As in the previous experiment the original volume of herbage in the silos was 3·02 cu. m. This fell rapidly in all silos until by the 3rd day the volumes were A, 1·31; B, 1·51; C, 1·10; and D, 1·20 cu. m. The volumes in silos A, C and D followed a similar pattern and at the end of the ensiling period were 0·94, 0·85 and 0·90 cu. m. respectively. The volume in B remained consistently higher throughout the experiment, the final value being 1·0 cu. m.

Composition

The composition of the grass and silages is given in Table I. The grass was low in dry matter (13·9–14·2%) and soluble carbohydrates (4·3%). The cocksfoot had been cut at a leafy stage of growth and was of high protein content. The silages made from long herbage were similar in pH value and organic acid content. They were not well preserved (pH 4·9) and were relatively low in lactic acid content. The silage obtained from silo D (bruised–inoculated) was of lowest pH value (4·2) and of highest lactic acid content. The bruised material was of higher pH value (4·5) and lower lactic acid content.

The graph in Fig. 1 shows the pH pattern of the effluents. The values all fell to 4·2 by the 9th day, but increased again gradually in the effluents from silos A and B. The pH values of the effluent from silo C fell to 4·1 by the 10th day, remained constant and did not increase until the end of the ensiling period.

In silo D the value fell to 4·0 by the 9th day and remained at this level during the experiment.

Temperature changes

The temperatures again remained relatively low, the maximum values occurring during the first 5 days. These were A, 18°; B, 20°; C, 19°; and D, 20°.

Losses

The total fresh weights of silage removed from silos A, B, C and D respectively were 798, 756, 704 and 697 kg. The corresponding surface waste material was 3·1, 4·1, 1·0 and 1·9% of the total silage removed.

Effluent weights collected over the ensiling period were A, 104; B, 144; C, 197; and D, 202 kg. The losses of dry matter and its components are shown in Table II. The gaseous dry matter losses were A, 7·2; B, 8·8; C, 2·1; and D, 1·5%. As in the previous experiment the gaseous nitrogen losses were small and did not exceed 4%.

Nutritive value

The digestibilities of the grass and silages are given in Table III. The slightly higher digestibility values for the silages, especially A and B, compared with the original herbage could be due to two factors. Firstly, abnormally high residues of silage were left in the feed-boxes, which could have indicated some selectivity. Secondly the silages in this trial were fed at voluntary intake levels, the mean daily dry-matter intakes (g./kg. $W^{0\cdot73}$) being: long grass, 56; bruised grass, 45; silages A, 24; B, 33; C, 28; and D, 45. The relatively low intakes of silages A, B and C may have influenced the digestibility.

Bacteriological studies

The untreated fresh grass in the second experiment yielded a low count of lactic acid bacteria which were mainly heterofermentative streptococci. A much greater number of organisms was obtained from the bruised material. In examinations of the laboratory-made silages, the Gram-negative bacteria were no longer found after 3 days of incubation. After 3 days the count on acetate agar was approximately the same in the untreated and the bruised material and had risen at the 8-day examination, whereas in both the inoculated silages the numbers had been higher at 3 days and then had declined. Pediococci were found in all the silages, being most numerous in the untreated material. They appeared to increase slightly between 3 and 8 days while streptococci seemed to decline in numbers. Anaerobes were detected in all the silages.

After 160 days the large silos provided material which yielded a high count on acetate agar. There was little difference between counts from the upper and lower layers of the silage. Anaerobes were few in number. They were mainly proteolytic types and were slightly more numerous in the bruised–inoculated material.

Discussion

In the first experiment the ryegrass ensiled was relatively rich in soluble carbohydrates (16·2%), but in spite of this the addition of molasses reduced the gaseous dry-matter losses. It is likely that with long herbage the sugars are not readily available to the bacteria and lactic acid production cannot occur until the plant cells collapse and liberate their contents. Results with the laboratory silos indicated that the addition of molasses stimulated the growth of the lactobacilli, streptococci and pediococci which were present initially on the fresh herbage. Although the inoculum appeared to have no beneficial effect in reducing gaseous losses, there is evidence, from the effluent pH graph and from the results of the laboratory experiment, that the pH values fell more rapidly in the inoculated–molassed herbage than in the molassed material alone. The effect of the bacterial culture was more noticeable in the tube 'silos' where the resultant silages which had been inoculated were of markedly lower pH value than the ordinary material. It is possible that the temperature differences between tube and field silos may have been an important factor in the contrasting results. The count of bacteria in the large silos gives little information concerning the course of the bacterial development beyond indicating the final dominance of lactic acid bacteria. The effects of varying temperatures and pH values during the fermentation in the large silos result in bacterial counts which are higher than those finally obtained from the tube silages which were held under controlled conditions.

In spite of the low content of dry-matter of the ryegrass, all silages were well preserved (pH 3·9–4·1). It is well known that wet herbage is more difficult to ensile satisfactorily than herbage of relatively high dry-matter content, but the disadvantages of wet herbage can, in part, be offset by the use of molasses as an additive. It is possible that the relatively high soluble carbohydrate content of the original herbage was an important factor in producing a satisfactory fermentation.

In the second experiment, cocksfoot of low content of soluble carbohydrate (4·3%) was used. Here, the initial lactic acid bacteria population of the bruised herbage was higher than that of the long material. Stirling & Whittenbury[10] have already shown that, although small numbers of lactic acid bacteria occur frequently on the growing plant, they are especially numerous on machines that chop, bruise or lacerate the crops and appear to accumulate where plant sap tends to collect.

A surprising feature of the bruised silages was their relatively high lactic acid content (5·8–7·4%) in view of the low soluble carbohydrate content of the original herbage. It is clear that lactic acid had been produced from compounds other than sugars and fructosans, possibly from organic acids, from pentoses resulting from the hydrolysis of hemicelluloses or from amino-acids. Organic acids in the original herbage were not determined in these studies but it is known, for example, that many lactobacilli can dissimilate malate with the production of lactic acid.[11]

Chromatographic examination of the herbage and silage extracts indicated that hydrolysis of hemicelluloses had occurred. In silo D it was estimated that sugar production from this source was of the order of 4%. Even if this were all available to the bacteria as an energy source, the lactic and volatile acids (11%) were in excess of the total sugars that could be made available. It is however difficult to relate values for percentage composition of the herbage and silages to actual chemical changes.

The gaseous dry-matter losses from silos C and D were extremely low compared with those from silos A and B, suggesting that very little respiration had occurred in the bruised material. It is well known that respiration losses are largely related to the degree of compaction and it is clear from the volume results quoted earlier that silos C and D compacted more readily than A and B. With herbage of low content of soluble carbohydrate, bruising has an obvious advantage in preventing respiration and thereby conserving sugars for acid production by bacteria.

Perhaps the most surprising feature about the second experiment is that well-preserved material was produced in the bruised, inoculated material in spite of the high moisture and low soluble carbohydrate contents.

From these results it seems that no advantage is gained by inoculating a material relatively rich in soluble carbohydrates, such as fairly mature ryegrass. Practical measures such as increasing the dry-matter content by wilting, bruising to make the plant juices available and compacting adequately in order to exclude air from the silo are of greater importance with material of this type.

With crops which are frequently low in soluble carbohydrates such as cocksfoot, or have high buffering properties such as clovers and lucerne, the addition of molasses and an inoculum may prove to be advantageous. In such cases it is important that the available soluble carbohydrates are utilised efficiently for the production of lactic acid. An early dominance of homofermentative lactic acid bacteria could prove critical in achieving a satisfactory type of preservation. These organisms can produce, from the same amount of carbohydrate, twice as much lactic acid as the heterofermentative types, which may frequently become dominant in uninoculated silage.

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DETERMINATION OF WATER-SOLUBLE CARBOHYDRATES IN GRASS

By P. McDONALD and A. R. HENDERSON

A simple method involving a single extraction procedure is given for the determination of water-soluble carbohydrates (sugars + fructosans) in grasses. Factors such as storage of grass samples and clarification, hydrolysis and storage of extracts are discussed. The method is likely to have a routine application in silage studies where a measure of fermentable carbohydrates is required.

Introduction

The carbohydrates extracted from grass with cold water include the monosaccharides, glucose and fructose; the oligosaccharides, sucrose, melibiose, raffinose and stachyose; and fructosans.[1] The importance of these water-soluble carbohydrates (WSC), both as a readily available supply of energy to rumen micro-organisms and as a source of lactic acid in silage making, has been stressed by many workers.

In a number of silage studies carried out at Edinburgh,[2-4] the determination of WSC in herbage samples has consisted of a two-fraction process involving extractions with ethanol and cold water based on the method devised by Wylam.[5] This procedure gives separate values for sugars and fructosans. Since fructosans are readily available to many lactic acid bacteria,[6] there seems little justification in determining these carbohydrates separately in silage studies, particularly as a more rapid routine method is desirable for handling large numbers of samples.

The purpose of this study was to devise a simple method involving a single extraction which would enable the WSC in herbages to be determined as a routine procedure.

Experimental and results

Analytical procedure

The method devised for the determination of WSC in fresh grass samples was as follows: Twenty-five-g. samples of the chopped grass are macerated with 200 ml. of water in a top-drive macerator for 5 min. The fibrous residue is removed by filtration through cloth and 5 ml. of 0·1N-sulphuric acid are added to 100 ml. of the filtrate in a conical flask. The acidified extract is brought to boiling point in order to coagulate proteins and if necessary Celite is added to aid filtration. The proteins are removed by filtration (Whatman No. 1) and the residue is washed with distilled water. The filtrate is made up to 250 ml. Hydrolysis is effected by adding 5 ml. of 2N-sulphuric acid to 15 ml. of the diluted filtrate in a boiling tube fitted with a cold-finger condenser and immersing the tube in a boiling water bath for 10 min. After being cooled for 5 min. the hydrolysate is made neutral to methyl red with sodium hydroxide and made up to volume (50–100 ml.). Aliquots of 5 ml. are analysed for reducing sugars by the modified Somogyi reagent of Wiseman *et al.*[7]

To test the reproducibility of the method, determinations were carried out on two herbages. On 6 samples of *Lolium perenne*, the WSC were 27·8% (S.E. $\pm$0·11) and on 4 samples of a *Festuca pratensis–Phleum pratense* mixture the WSC were 12·0% (S.E. $\pm$0·06). In addition the WSC of 6 separate samples of *Lolium perenne* with sucrose added at the time of maceration were determined and the results are given in Table I.

Table I

Recoveries of added sucrose [WSC values of L. perenne *(g./25 g. grass) + 1 g. sucrose]*

	Theoretical value *a*	Analytical value *b*	$\frac{b}{a} \times 100$
1	2·358	2·274	96·4
2	2·358	2·314	98·1
3	2·358	2·371	100·6
4	2·358	2·358	100·0
5	2·358	2·346	99·5
6	2·358	2·383	101·1

Preparation and extraction

Sugars and fructosans play an important part in the photosynthetic and respiratory processes occurring in plants and may undergo rapid changes immediately after cutting. Unless fresh samples can be analysed immediately, it is important that the enzyme systems in the plant be completely inactivated. Several methods for doing this have been proposed of which rapid drying is the commonest. A systematic investigation into methods of drying and preservation of ryegrass for subsequent analysis was undertaken by Collins & Shorland[8] who concluded that air drying did not arrest changes in free-sugar content on storage. Wylam[9] has stated that there is a risk of destruction of sugars during drying, while Waite & Boyd[10] stressed the need for rapid drying and, in their studies, dried fresh herbage in a forced-draught oven and ground within ¾–1 h. from the time of cutting. Deriaz[11] has claimed that drying in a Unitherm oven at 100° caused little loss of soluble carbohydrates, although he did not comment on changes occurring during prolonged storage of dried samples. Perhaps the most certain method of arresting enzyme activity is to immerse the freshly cut sample into boiling ethanol.[9] This procedure, however, is tedious and difficult when handling large numbers of samples. An alternative method of storing fresh samples is deep freezing and in the present studies the effectiveness of deep freezing samples (approx. 1 kg.) at $-16°$ on changes in WSC was examined.

Results for three samples (Table II) indicated that WSC values increased by 4–14% during storage for 24 h. It would appear from these results that enzymic action is not immediately arrested and this may be in part due to the difficulty in obtaining a rapid temperature drop in herbages stored in bulk. Evidence has been obtained which shows this to be the case. An experiment was carried out in which different quantities of grass contained in polythene bags (38×76 cm., maximum capacity 16 l.) were stored at $-16°$. The temperature at the centre of a 500-g. sample fell to 0° after 2 h. and to $-14°$ after 24 h. The corresponding temperatures for a 3000-g. sample were 17° and $-1°$. After 48 h. the temperature of the larger sample had fallen to $-12°$.

In an attempt to achieve a rapid freezing, small quantities (25 g.) of grass were stored under similar deep-freeze conditions in small polythene bags containing solid CO_2. This procedure did not prevent, however, an increase in WSC (Table II).

Some workers have recommended the use of hot water for the extraction of carbohydrate, but there is evidence that hot water removes pectin and some hemicelluloses in addition to sugars and fructosans from herbage.[12] Wylam[5] has found that both galactose and arabinose are present in ryegrass extracts at 50°, whereas only a very slight trace of araban is extracted with cold water. Since it has been shown that hemicelluloses are not attacked by lactic acid bacteria,[13] the presence of these carbohydrates in water extracts falsifies the fermentable carbohydrate content in silage studies. In view of these findings it was decided to use a cold-water extraction procedure in these studies. The type of macerator used for the preparation of extracts is important since it was found that certain bottom-drive macerators tended to heat the mixture.

Clarification

Laidlaw & Reid[14] have examined a number of procedures for clarifying alcoholic extracts, and concluded that barium hydroxide–cadmium sulphate was the most suitable. Clarification, however, involving the production of large precipitates has been shown in our studies with water extracts to hold back some fructosans. A simple method of clarifying water extracts of grass is to precipitate protein material by heat coagulation,[15] which is most efficiently carried out about pH 4·5.

Although some proteins are soluble in boiling water, Pirie[16] has stated that these do not occur sufficiently widely to be a frequent cause of error. Amino-acids are known to interfere with the reductometric determination[17] but these are not usually present in sufficiently large amounts in grass to cause error.

In order to determine whether interfering substances were present in the clarified water extracts, aliquots were treated with ion-exchange resins [IR-120 (H) and IR-45 (OH)] and activated charcoal. WSC values were similar to those obtained for extracts which had not been treated.

Hydrolysis

Wylam[5] has shown that hydrolysis of oligosaccharides with 0·5N-sulphuric acid requires 4 h. for completion. Unfortunately this treatment destroys appreciable amounts of fructose and a correction involving a separate fructose determination is necessary. Since the oligosaccharides, excluding sucrose, constitute a small part of the WSC fraction[18] and a 10 min.

Table II

Changes in WSC during storage of grass (approx. 1 kg.)

(% WSC in dry matter)

		Before	After storage	Increase, %
1	*Lolium perenne* after 72 h.	22·3	24·7	10·8
2	*Dactylis glomerata* after 24 h.	9·8	10·2	4·1
3	*Festuca pratensis–Phleum pratense* mixture after 24 h.	12·0	13·7	14·2
4	*Lolium perenne* + CO_2 after 24 h.	12·0	13·7	14·2

hydrolysis with 0·5N-sulphuric acid has been shown to hydrolyse completely sucrose and fructosans with a loss of only 1·2% fructose, it was decided to adopt the shorter period of hydrolysis.

Determination

The Somogyi reductometric method[19] has been used in grass and silage investigations satisfactorily for a number of years. The reagent as modified by Wiseman *et al.*[7] has the advantage with mixed sugar extracts that glucose and fructose have the same titration factors.

Comparison of the method with the ethanol/water extraction procedure

The method described above was compared with the two-fraction procedure of Wylam[5] described in earlier publications.[2–4] The results of these two methods are given in Table III.

Table III

WSC (% of dry matter) of fresh grass samples determined by two different methods

	Ethanol/water extraction						Water extraction only
	4 h. hydrolysis			10 min. hydrolysis			10 min. hydrolysis
	Sugars	Fructosans	Total	Sugars	Fructosans	Total	Total
L. perenne	15·6	7·2	22·8	15·2	7·2	22·4	22·3
D. glomerata	12·6	2·0	14·6	12·6	2·0	14·6	14·9
F. pratensis–P. pratense mixture	10·3	2·1	12·4	9·7	2·1	11·8	12·0

In the ethanol/water extraction method, an aliquot of the ethanol extract was hydrolysed for 4 h. with 0·5N-sulphuric acid to hydrolyse completely the oligosaccharides and a correction applied for fructose decomposition, while a second aliquot was hydrolysed for only 10 min. as in the new procedure described earlier. The results suggest that the 10-min. hydrolysis is sufficient for routine purposes. The results in the final column obtained by the new method agree reasonably well with those obtained by the ethanol/water extraction procedure.

Storage of extracts

Where large samples of fresh grass are handled, it may not be convenient to complete the analysis in one day and it may be necessary to store extracts. The most suitable stage at which to do this is after removal of the fibrous material. It was found that extracts stored after being boiled in sterile tubes and kept between chloroform and toluene at −16° over a period of 50 days, gave WSC values similar to those for the fresh extracts.

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Hence, a unit increase in T_A means a decrease in S_A of only 0·287 units. It is, therefore, concluded that, in a young potato plant, an internal factor associated with surplus carbohydrate restricts photosynthesis until tubers develop to form a sink for carbohydrate and remove the restriction on E.

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FERMENTATION STUDIES ON RED CLOVER

By P. McDONALD, ANNA C. STIRLING, A. R. HENDERSON and R. WHITTENBURY

Two experiments were carried out to study the fermentation changes occurring during the ensilage of red clover (*Trifolium pratense*). In the first experiment red clover containing 11·8% of water-soluble carbohydrates (WSC) was bruised and ensiled, the treatments being: control, molassed, inoculated, and wilted. All silages were well preserved (pH 3·9–4·0), the gaseous losses being lowest in the control and inoculated herbages.

In the second experiment the effect of bruising red clover before ensiling was studied. Two silos were filled with long material (WSC—9·7%) and two with bruised material (WSC—8·0%). The bruised silages were well preserved (pH 4·0–4·1) and the bruising treatment resulted in lower gaseous losses than those which occurred in the production of the non-bruised silages (pH 4·3).

The results show the advantages of bruising clover and indicate that wilting, addition of molasses and inoculation of the clover may be advantageous. Feeding trials, using sheep, were carried out to determine digestibilities and intakes and the importance of the results is discussed.

Introduction

The advantages of legumes as rich sources of protein and essential mineral elements, especially, calcium and magnesium, in the diets of ruminants are well known. Unfortunately legumes have usually been regarded as difficult crops to conserve, especially as silage. This difficulty has been attributed to their low content of water-soluble carbohydrates (WSC)[1,2] and to their relatively high buffering capacity compared with grasses.[3-5] Of the legumes lucerne has been studied fairly extensively[6,7] and the use of molasses as an additive in silage making has been recommended. In other studies with unwilted legumes, the addition of molasses improved the fermentation process but resulted in high seepage losses of sugars.[8]

Recent studies on different varieties of red clover (*Trifolium pratense*) have shown that yields of up to 12,000 lb. of dry matter/acre can be obtained in S.E. Scotland,[9] and it is suggested that this crop may have considerable value in grassland economy under Scottish conditions.

The purpose of the present investigation was to study the effect of a number of treatments upon the biochemical changes and losses during the ensilage of red clover.

Experimental

Procedure

The silo unit used in these studies consisted of four metal silos (153 cm. dia. $\times$ 182 cm. high) each suspended from a weighing device which enabled daily measurements of weight changes to be recorded. Each silo was fitted with ten thermocouples which were sited at different levels within the herbage during filling. A detailed description of the silo unit and the techniques of sampling, digestibility and bacteriological methods have been given elsewhere.[10,11] The technique used for the separation and determination of organic acids was that developed by Lessard.[12] The acids are eluted from a silica gel column with benzene-butanol, collected in 4-ml. fractions and titrated with 0·005N-sodium hydroxide. Dry-matter contents of silages were determined by a toluene distillation technique.[13] Water-soluble carbohydrates were determined after extraction by a modified Somogyi technique.[14] The buffering capacity to lactic acid of the herbage samples was determined as described by McDonald & Henderson[5] and the results were designated 'lactic buffer capacity (LBC)', i.e., mg. of lactic acid required to bring 1 g. of dried, milled herbage to pH 4. Cellulose was determined by the Crampton & Maynard method.[15]

In order to assess the true losses occurring during the formation of surface waste, a technique based on that described by Wittwer *et al.*[16] was employed. When the silos were three-quarters full, a sheet of fine mesh Terylene netting was placed on the surface of the herbage to act as a marker. Above this were placed four perforated polythene bags containing weighed samples (4 kg.) of herbage representative of the mass. The silos were filled as before and the weight of herbage above the net recorded. When the silos were emptied, the waste material, polythene bags and well-preserved silage above the netting were separately weighed. From these recordings, together with dry-matter values, the losses occurring during the production of the waste material could be estimated.

First experiment.—Broad red clover (*Trifolium pratense*) was used and the treatments were as follows:

Silo A: clover 1678 kg. (238·4 kg. of dry matter)
Silo B: clover 1500 kg. (213·2 kg. of dry matter) + molasses solution 72·6 kg. (33·9 kg. of dry matter)
Silo C: clover 1671 kg. (237·4 kg. of dry matter) + inoculum 4 kg. (0·6 kg. of dry matter)
Silo D: partially wilted clover 1551 kg. (312·4 kg. of dry matter)

In the first three treatments, the clover was cut and bruised on 24 June, 1963, with a flail-type forage harvester (Lundell 60, rotor speed 1,200 r.p.m.) and ensiled the same day. In treatment D, the clover was cut with a mower on the morning of 24 June, wilted for 27 hours, then lifted and bruised with the flail-type forage harvester.

The molasses used in treatment B consisted of 48·4 kg. of molasses diluted with 24·2 kg. of water and this was sprayed on to the clover from a pressure spray during filling. The molasses contained 70·1% of dry matter, 0·27% of total nitrogen, and 44·8% of WSC.

The inoculum consisted of a suspension of lactobacillus strains selected for their ability to grow rapidly in silage and to ferment all of the available carbohydrates.

In addition to the large silo experiments, a number of small laboratory silos were filled with similar material and were subsequently examined at intervals for bacteriological changes.

After filling, the contents of silos A, B and C occupied 3·02 cu.m., and silo D, 3·40 cu.m. The consolidation weight applied to the surface of the ensiled material was 400 kg., equivalent to a pressure of 22 g./sq.cm.

Effluents were collected daily, or when they appeared, and stored at $-18°$ until subsequently analysed. The silos were opened on 20 August, 58 days after filling.

Digestibility trials and intake measurements were carried out on the fresh clover in triplicate and on the silages in duplicate using Cheviot wether sheep.

Second experiment.—This was designed to study the effect of bruising. The experiments were carried out in duplicate as follows:

Silo A: long clover 910 kg. (151·6 kg. of dry matter)
Silo B: long clover 910 kg. (151·6 kg. of dry matter)
Silo C: bruised clover 910 kg. (140·0 kg. of dry matter)
Silo D: bruised clover 910 kg. (140·0 kg. of dry matter)

The clover was a second cut taken on 18 September, 1963, from the same field as that used in the first experiment, but whereas the June cut consisted almost entirely of red clover, the material used in the second experiment contained about 34% of grasses, mainly *Lolium perenne*.

The long herbage was cut with a mower and hand loaded. The bruised material was cut and bruised with a flail-type forage harvester similar in make and type to that used in the first experiment. All four silos were filled with the fresh herbage on the same day.

The contents of silos A and B occupied 3·02 cu.m. while the bruised material occupied 2·27 cu.m. after filling. The consolidation weight applied was 400 kg. as in the first experiment. Similar procedures to those described above were adopted for measurements of waste and digestibility. The silos were opened on 19th November, 63 days after filling.

Results

(1) *Experiment* 1

Volume changes

The changes in volume occupied by the silages are shown in Fig. 1. The volume of the molassed herbage fell most rapidly, while that of the wilted material remained consistently higher throughout the ensiling period; the latter had however a greater weight of dry matter per unit volume at the end of the experiment.

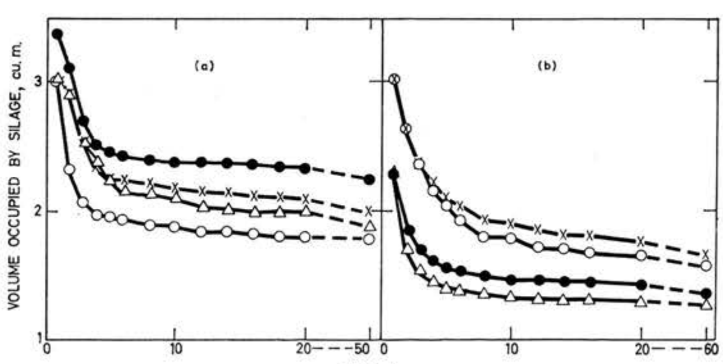

FIG. 1.—*Volumes occupied by silages at various times*

(a) Expt. 1 (b) Expt. 2 Treatment: A × B ○ C △ D ●

Composition

The chemical composition of the clover and silages is given in Table I. The fresh clover was of low dry-matter content (14·21%). At the time of cutting, wilting conditions were poor and, after 27 h., the dry matter content was only 20·14%.

Table I

Composition of clovers and silages (% of true dry matter)

	Experiment 1						Experiment 2					
	Clover		Silages				Clover		Silages			
	Fresh	Wilted	A	B	C	D	Long	Bruised	A	B	C	D
Dry matter[a]	14·21	20·14	17·72	18·73	17·48	19·66	16·66	15·38	16·56	16·97	18·89	18·95
Organic matter	89·3	87·3	88·8	90·5	90·3	86·3	90·6	85·6	89·8	89·9	81·2	83·7
Crude protein	15·1	13·7	14·7	13·9	15·6	14·8	14·9	15·2	18·3	17·3	15·8	14·9
Ether extract	2·7	2·4	2·5	2·0	2·3	2·3	2·2	2·1	2·9	2·9	2·4	2·2
Crude fibre	24·3	23·8	27·3	26·6	26·8	25·0	25·1	23·1	26·4	26·7	24·2	24·8
N.F.E.[b]	47·3	47·5	44·3	47·9	45·6	44·2	48·3	45·3	42·2	43·0	38·8	41·9
Total N	2·41	2·19	2·35	2·23	2·49	2·37	2·39	2·43	2·93	2·77	2·53	2·38
Protein N	1·88	1·76	1·07	1·00	1·17	1·23	1·99	2·03	1·57	1·51	1·71	1·59
Non-protein N	0·53	0·43	1·28	1·23	1·32	1·14	0·40	0·40	1·36	1·26	0·82	0·79
Volatile N	0·02	0·02	0·20	0·15	0·18	0·19			0·26	0·26	0·12	0·12
Water-soluble carbohydrates	11·8	11·2	0·7	2·9	0·7	0·6	9·7	8·0	1·6	1·4	1·4	1·2
Cellulose	28·9	28·5	31·8	30·1	32·3	29·4	28·6	27·6	29·9	30·5	28·8	29·2
Lignin	7·0	6·9	7·9	7·1	8·7	7·8	9·5	9·5	9·8	9·9	9·5	10·1
Lactic acid			9·3	12·4	10·4	10·5			6·7	6·8	7·9	7·6
Formic acid			nil	nil	nil	nil			nil	nil	nil	nil
Acetic acid			3·6	2·7	2·5	2·9			2·5	2·7	2·5	2·4
Propionic acid			nil	0·49	0·26	0·20			nil	nil	nil	nil
Butyric acid			0·08	nil	nil	nil			nil	nil	nil	nil
Succinic acid			0·14	0·30	nil	0·21			0·40	nil	nil	nil
pH[a]	5·7	5·7	4·0	3·9	3·9	4·0	6·1	6·0	4·3	4·3	4·1	4·0

[a] = determined on fresh material [b] N.F.E. = Nitrogen-free extractives

The WSC content of the fresh herbage was 11·8% and that of the wilted material slightly lower (11·2%). The four silages were well preserved, the molassed and inoculated materials being of slightly lower pH value than the control and wilted silages. The lactic acid (9·3–12·4%) and acetic acid (2·5–3·6%) contents were relatively high, the highest lactic value occurring in the molassed silage. Butyric acid was detected, in trace amounts, in the control.

The LBC value of the fresh clover was 63 and that of the wilted 58.

The effluent pH values for the control and wilted materials are given in Fig. 2. The values for the effluents obtained from the molassed and inoculated herbages followed a similar pattern to those of the control.

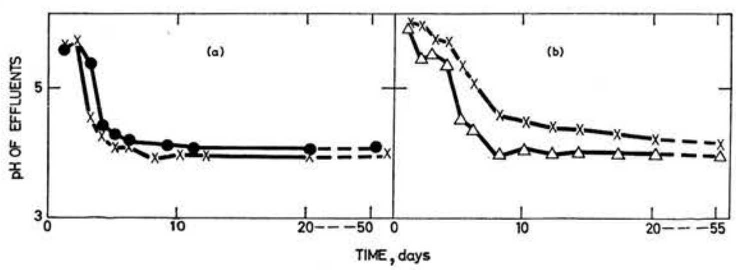

FIG. 2.—*pH values of effluent after various times*

(a) Expt 1. (b) Expt. 2 Treatment: A × C △ D ●

Temperature changes

The temperature in all four silos remained relatively low, the maximum values reaching 20° (A), 22° (B), 21° (C) and 22° (D). The temperatures in silo D remained slightly higher than those in the other three silos during the whole of the ensiling period.

Losses

The total weights of fresh silage removed from the silos and the total losses of dry matter and its components are shown in Table II.

Table II

Percentage losses during ensilage

	Experiment 1								Experiment 2							
	A		B		C		D		A		B		C		D	
	Total	Effluent	Total	Effluent	Total	Effluent	Total	Effluent	Total	Effluent	Total	Effluent	Total	Effluent	Total	Effluent
Total fresh	30·0	29·1	33·2	32·2	29·9	29·3	11·1	10·4	14·2	13·1	15·3	14·1	25·3	24·6	25·3	25·0
Dry matter	13·9	9·0	21·1	14·0	14·4	9·4	13·9	3·2	16·1	2·6	16·3	2·8	9·2	7·2	9·5	6·9
including																
gaseous dry matter	4·9		7·1		5·0		10·7		13·5		13·5		2·0		2·6	
dry matter in waste material	36·5		38·2		43·6		51·5		35·2		33·4		37·8		23·8	
dry matter in well-preserved material	11·6		19·9		11·9		11·3		14·2		13·4		4·4		7·6	
Crude protein	16·0	10·3	17·5	11·9	11·6	11·5	6·6	4·2	+2·9	3·7	3·1	4·0	5·4	7·0	12·9	7·0
Ether extract	21·3		31·9		28·5		19·0		+7·4		+10·7		+3·1		5·2	
Crude fibre	3·4		nil		5·6		9·2		11·7		11·1		4·9		2·6	
N.F.E.[a]	19·2		28·0		17·4		20·0		26·7		25·5		22·1		16·3	
Total N	16·0	10·3	17·5	11·9	11·6	11·5	6·6	4·2	+2·9	3·7	3·1	4·0	5·4	7·0	12·9	7·0
Protein N	50·9		58·1		46·8		39·8		33·8		36·4		23·6		29·2	
Water-soluble carbohydrates	94·7	10·5	88·0	23·5	95·0	8·5	95·5	3·0	86·3	2·7	88·1	2·3	83·9	17·3	86·9	17·2
Cellulose	5·0		4·5		4·1		11·2		12·3		10·8		5·3		4·1	
Lignin	2·1		7·4		+6·3		1·9		13·5		12·8		8·9		3·4	
Water	32·7	32·4	35·5	35·6	32·4	32·6	10·5	12·2	13·8	15·2	15·2	16·4	28·2	27·8	28·2	28·3

[a] see Table I + means increase instead of loss

The quantities of surface waste material removed from the silos were A, 94; B, 64; C, 71; and D, 59 kg. From the buried bag and marker technique the partition of losses of dry matter between this waste material and the well preserved material were estimated and these are also given in Table II.

Nutritive value

The digestibility coefficients and digestible nutrients for the clover and silages are given in Table III. During the trials the sheep were fed at voluntary intake levels. The daily dry matter intakes (g./kg.$W^{0.73}$) are also shown in Table III.

Bacteriological studies

Most of the bacteria on the fresh clover were Gram-negative types but the number of lactobacilli was appreciable, 90,000/g. In the small laboratory-made silages, examined after 4 days, the count of lactic acid bacteria was highest in the molassed and inoculated silages and lowest in the wilted material. After 9 days, lactobacilli were dominant in all the silages by which time the count on the wilted material had risen to the level found in the inoculated silage at the four-day examination. The pH values were all low; A (control), 4·2; B (molassed), 4·1; C (inoculated), 3·8 and D (wilted), 4·1. After 46 days, with lactobacilli the surviving organisms, the pH values were, A, 4·3; B, 4·0; C, 4·1 and D, 4·2. The most rapid drop in pH occurred in the inoculated material. The possibility that the tendency for a rise in pH might be due to a limiting sugar content is suggested by the continued decrease in pH value found in the molassed silage. As would be expected, bacterial action in the wilted material was slow, although the number of naturally occurring lactobacilli on the fresh crop was sufficient to ensure that they became dominant in all the laboratory silages.

When the large experimental silos were opened, lactobacilli were again found to be dominant in all silages.

(2) *Experiment* 2

Volume changes

The long herbage was more difficult to compact than the bruised material and the volumes in silos A and B remained consistently higher than those in silos C and D (Fig. 1).

Composition

It can be seen from Table I that the dry matter contents of the clover were again low (15·38, 16·66%). The WSC values were 9·7 and 8·0% for the long and bruised herbages respectively. All silages were well-preserved, but the bruised materials were of lower pH value and of slightly higher lactic acid content than the long herbage. Butyric acid was not detected in any of the samples.

The LBC value of the long herbage was 54 and that of the bruised material 55.

Analysis of the effluents indicated that the pH values of the bruised silages fell more rapidly than those of the long material. The results of treatments A and C are shown in Fig. 2.

Temperature changes

The temperature in the silos again remained low, the maximum values being A, 22°; B, 18°; C, 20° and D, 19°.

Losses

The total weights of fresh silage removed from the silos and the total losses of dry matter and its components are shown in Table II.

The quantities of surface waste material removed from the silos were A, 76; B, 110; C, 73; and D, 77 kg. As in the previous experiment, the partition of losses of dry matter between the waste and the well preserved material were estimated from the buried bag and marker technique and the results are shown in Table II.

Table IIIA

Percentage digestibility (D) *and percentage of digestible nutrients* (DN) *in true dry matter*

	Clover		Silages A		Silages B		Silages C		Silages D	
	D	DN	D	DN	D	DN	D	DN	D	DN
Experiment I										
Organic matter										
1.	72·7	65·0	67·9	60·3	73·6	66·6	70·6	63·8	68·4	59·0
2.	73·7	65·8	72·0	63·9	70·0	63·4	70·2	63·4	70·0	60·4
3.	70·2	62·7								
Mean	72·2	64·5	70·0	62·1	71·8	65·0	70·4	63·6	69·2	59·7
Crude protein										
1.	65·5	9·9	61·2	9·0	64·4	9·0	65·9	10·3	62·0	9·2
2.	69·6	10·5	69·8	10·3	61·8	8·6	66·5	10·4	66·8	9·9
3.	66·8	10·1								
Mean	67·3	10·2	65·5	9·7	63·1	8·8	66·2	10·4	64·4	9·6
Ether extract										
1.	57·5	1·6	54·6	1·4	47·0	1·0	49·4	1·1	57·1	1·3
2.	62·8	1·7	66·0	1·6	45·0	0·9	52·8	1·2	59·1	1·3
3.	58·0	1·6								
Mean	59·4	1·6	60·3	1·5	46·0	1·0	51·1	1·2	58·1	1·3
Crude fibre										
1.	68·3	16·6	65·4	17·9	73·5	19·6	67·7	18·2	64·0	16·0
2.	66·9	16·3	69·4	19·0	68·2	18·2	66·6	17·9	64·9	16·2
3.	60·8	14·8								
Mean	65·3	15·9	67·4	18·5	70·9	18·9	67·2	18·1	64·5	16·1
N.F.E.[a]										
1.	78·2	36·9	72·5	32·1	77·5	37·1	75·0	34·2	73·6	32·5
2.	79·1	37·4	74·6	33·1	74·5	35·7	74·4	33·9	74·6	32·9
3.	76·8	36·3								
Mean	78·0	36·9	73·6	32·6	76·0	36·4	74·8	34·1	74·1	32·7
S.E.[b]										
1.	—	58·7	—	53·1	—	59·2	—	56·4	—	52·3
2.	—	59·7	—	56·9	—	55·9	—	56·0	—	53·8
3.	—	56·5								
Mean	—	58·3	—	55·0	—	57·6	—	56·2	—	53·1
Experiment II										
Organic matter										
1.	67·7	58·0	65·8	59·1	66·5	59·8	65·5	53·2	67·4	56·4
2.	68·4	58·5	64·5	57·9	67·6	60·8	67·5	54·8	67·3	56·4
3.	69·3	59·3								
Mean	68·5	58·6	65·2	58·5	67·1	60·3	66·5	54·0	67·4	56·4
Crude protein										
1.	62·3	9·5	67·9	12·4	67·4	11·7	63·5	10·1	60·4	9·0
2.	64·6	9·8	68·1	12·5	66·7	11·6	62·2	9·9	60·1	9·0
3.	63·3	9·6								
Mean	63·4	9·6	68·0	12·5	67·1	11·7	62·9	10·0	60·3	9·0
Ether extract										
1.	66·8	1·4	59·3	1·7	65·8	1·9	71·2	1·7	67·2	1·5
2.	67·6	1·4	64·0	1·8	60·3	1·8	68·9	1·6	64·4	1·4
3.	62·6	1·3								
Mean	65·7	1·4	61·7	1·8	63·1	1·9	70·1	1·7	65·8	1·5
Crude fibre										
1.	62·2	14·3	63·4	16·8	62·7	16·7	62·6	15·1	66·0	16·4
2.	62·0	14·3	58·9	15·6	67·4	18·0	66·0	15·9	65·9	16·3
3.	65·4	15·1								
Mean	63·2	14·6	61·2	16·2	65·1	17·4	64·3	15·5	66·0	16·4
N.F.E.[a]										
1.	72·4	32·8	66·9	28·2	68·5	29·5	67·8	26·3	70·6	29·6
2.	72·9	33·0	66·4	28·0	68·6	29·5	70·5	27·4	70·9	29·7
3.	73·5	33·3								
Mean	72·9	33·0	66·6	28·1	68·6	29·5	69·2	26·9	70·8	29·7
S.E.[b]										
1.	—	52·0	—	52·3	—	53·1	—	47·1	—	50·0
2.	—	52·5	—	51·2	—	54·0	—	48·7	—	49·9
3.	—	53·2								
Mean	—	52·6	—	51·8	—	53·6	—	47·9	—	50·0

[a] N.F.E. = See Table I [b] S.E. = starch equivalent

Table IIIB

Daily intakes of dry matter
(g./kg. $W^{0\cdot73}$ where W = weight of sheep)

	Clover	Silages			
		A	B	C	D
		Experiment I			
1.	88·8	86·4	63·3	71·9	75·7
2.	93·3	69·7	71·9	74·2	65·0
3.	83·2				
Mean	88·4	78·1	67·6	73·1	70·4
		Experiment II			
1.	82·4	54·8	68·4	68·4	55·5
2.	92·1	60·7	58·3	56·2	59·3
3.	82·9				
Mean	85·8	57·8	63·4	62·3	57·4

Nutritive value

The digestibilities and daily dry matter intakes (g./kg. $W^{0\cdot73}$) of the clover and silages are shown in Table III.

Bacteriological studies

The initial count of lactic acid bacteria on the long herbage was 210/g. of fresh material, but, as might be expected through the release of plant juices and distribution of the organisms by mechanical action, it was considerably higher (18,000/g.) on the bruised material. When the silos were emptied, lactobacilli were dominant in all silages. The majority were homofermentative types which were able to decarboxylate malate. The growth of anaerobes was not extensive and the greatest number of proteolytic types were found in silo A.

Discussion

The WSC contents of the clovers, although low compared with those of *Lolium* spp.,[10,11] did not appear to be limiting in achieving a satisfactory type of fermentation in the experimental silos.

It is clear from the results of the first experiment that the addition of molasses had a beneficial effect in increasing the lactic acid content of the silage, although the inoculation treatment also resulted in material of similar pH value. The addition of molasses did increase the effluent production, however, and the total loss of dry matter from this treatment was higher than that from the other three. This confirms the findings of Wittwer *et al.*[8] It appears that, in practice, the addition of molasses to a very wet crop is liable to be wasteful because of the high effluent flow.

The buffering capacity of herbage is another important factor in ensilage. The LBC values were 58–63 and 54–55 in Experiments 1 and 2 respectively. These values are similar to those reported for legumes in an earlier publication[5] and are much higher than the values found for grasses.

Of great importance are the changes occurring during the early stages of fermentation, in which buffering and neutralising substances are increased through decarboxylation of organic acids such as malic acid. Legumes are richer in organic acids[17] than grasses and it is the breakdown of these substances by bacteria which adversely affects preservation.

The relatively low dry-matter content of the crop is one of the obvious disadvantages of ensiling red clover, and, in order to reduce losses of dry matter in the effluent, wilting seems to be an important pretreatment. This is clear from the effluent-loss figures for the partially wilted material ensiled in Experiment 1. Ideally, a dry-matter content of 25%, and preferably 30%, would be desirable. One fact, however, which is apparent from the first experiment is that the highest loss of gas was found with the wilted material. This may occur readily with wilted crops unless they are adequately consolidated. Wilting appears to be beneficial in reducing the buffering capacity. In Experiment 1, the LBC value of the wilted clover was 58 compared with a value of 63 for the fresh material. Smith[18] and Playne[19] have also commented on this reduction in buffering capacity after wilting herbage.

The benefit of bruising herbage before ensilage has been stressed by many workers[20,21] and this is clearly evident from the results of the second experiment.

The pH, as judged from the effluent samples (Fig. 2) fell more rapidly in the bruised than in the long material and the losses of dry matter were markedly lower in the forage harvested clover than in the long clover in spite of the higher effluent flow.

In these experiments the buried bag and marker technique, as described earlier, was used to measure losses of dry matter. It is clear from the calculated figures that the production of waste material leads to excessive losses of dry matter which are difficult to assess in farm practice based solely on surface waste measurement. The total losses of dry matter in the formation of waste material ranged from 36·5 to 51·5% in the first experiment and 23·8 to 37·8% in the second experiment.

The digestibilities of the silages are very similar to those of the fresh clovers. In Experiment II the digestible crude-protein figures of silages A and B are notably higher than those obtained for the other silages and the clover. These figures are a reflection of the crude protein percentages of these silages, which have increased as a result of the higher losses of gas during ensilage.

The intake figures are extremely variable and illustrate the need for carrying out feeding trials of this type with large numbers of animals. The values obtained, however, suggest that the silages were not consumed as readily as the fresh clovers.

The conclusions of these experiments indicate that red clover can be ensiled satisfactorily provided the herbage is bruised as a pretreatment. Because of the relatively high moisture content of this crop, wilting is also beneficial. The addition of molasses to red clover is not as important as when lucerne is ensiled, but may be an additional insurance against a butyric-type fermentation. Inoculation of clover crops, low in WSC, with homofermentative lactobacilli may be beneficial. A rapid fermentation would make the maximum use of the soluble carbohydrates as a potential source of lactic acid, cutting down sugar wastage by other bacteria which may dominate the microflora in crops with low numbers of homofermentative lactobacilli.

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A SILICA GEL CHROMATOGRAPHIC PROCEDURE ADAPTED TO LIQUID-SCINTILLATION COUNTING OF ^{14}C LABELLED ORGANIC ACIDS FROM PLANT MATERIAL AND SILAGE

By J. R. LESSARD* and P. McDONALD

A method is described for the separation of a wide range of organic acids present in plant material and in silage. The method consists of cold extraction with dilute sulphuric acid, followed by purification of the extract on a silica gel column. The organic acids in the purified extract were separated quantitatively using a continuous gradient-elution system of eluents through a small silica gel column.

The extraction method was found to be more efficient for extracting citric, oxalic and fumaric acids from red clover, than a standard method with ethanol, water and ion-exchange resins.

Most of the acids of the tricarboxylic acid cycle were detected in red clover. An unidentified acid accounted for more than 37% of the organic acidity.

The activity of ^{14}C-labelled acids could be measured directly on the eluate from the column in combination with a liquid scintillation system.

Introduction

In the course of a study of gaseous exchanges in ensilage, a method was sought which would be adaptable to the determination of both volatile and non-volatile organic acids in plant material and to the measurement at high efficiency of the radioactive carbon ^{14}C in the fractions obtained. Five main techniques, with a number of modifications, have been used for measuring organic acids, namely ion-exchange chromatography,[1,2] silica gel chromatography,[3] paper chromatography,[4] gas–liquid chromatography[5] and an older chemical method.[6] Of these, chromatography on silica gel seemed to be the procedure with the greatest scope, being adaptable to the separation of both volatile and non-volatile acids and also yielding acids in a relatively pure form.

Similarly there are numerous methods of extraction of acids from plant material, which include extraction into water, into ethanol and into ether. Of these, ether extraction is the only method not requiring a preliminary purification before the concentrated extract is separated through a silica gel column.

Isotopic ^{14}C-measurements in organic acids have been done routinely by using Geiger–Müller counters.[7,8] The method has the advantage of ease of preparation of samples but it also has a few serious disadvantages, such as low resolution and, in the case of ^{14}C-carbonates and -volatile acids, possibility of exchange of radioactive material with atmospheric CO_2. To our knowledge liquid-scintillation counting has not been used for counting ^{14}C-labelled organic acids directly on fractions eluted from a chromatographic column.

In the present study, a technique is described for the extraction of organic acids from plant material, and for their separation by silica-gel chromatography in a form suitable for ^{14}C-liquid scintillation counting.

Experimental

Samples for analysis

The samples of red clover were obtained from the 1963 crop grown on Langhill Farm of the University of Edinburgh, the crop being cut when it was just beginning to bloom. The crop was mainly red clover and the few grasses present were hand separated. A sample was oven-dried for determination of dry matter, which was found to be 14·5%. Fresh samples were stored in a freezer at —15° until required.

Extraction and purification methods

Two methods of extraction have been compared. (1) A 20-g sample was rapidly killed by dropping it into 100 ml of boiling ethanol and boiling was continued for 3 min. with a globe condenser on top of the beaker to prevent loss of volatile acids. The alcohol extract was decanted and saved and the residue macerated with cold 80% ethanol in a high-speed blender. The macerate was filtered and the residue was re-extracted twice with 80% ethanol, and then with distilled water with finally two water washings. The alcohol extract was concentrated to a syrup after adjustment to pH 8·5. The concentrated solution was filtered and mixed with the aqueous extracts and the combined extract purified by ion-exchange chromatography. It was first passed through a 10-ml column of Amberlite IR-120 (16–50 mesh) in the H^+-form to remove cations and amino-acids. The column was washed with 200 ml of distilled water. Precautions were taken to prevent the eluate from the column coming into contact with CO_2. An aliquot of the eluate was titrated to determine total anions and discarded. The remainder of the solution was passed through a second 12-ml column of Amberlite IR-45 in the OH^--form. The extract was followed by 150 ml of CO_2-free distilled water, and the acids were eluted with 300 ml of 4 N-aqueous ammonia. Ammonia was removed and the ammonium salts were concentrated to a small volume in a rotary evaporator at a temperature below 40°. The concentrate was desiccated over phosphorus pentoxide in a vacuum desiccator and the salts obtained were dissolved in sufficient water to make a total volume of 2 ml for application to the analytical column.

(2) The second method of extraction consisted in extraction with dilute acid.[3,9] A 20-g sample of finely chopped material was placed in a small wide-mouthed bottle and covered with 20 ml of 0·3 N-sulphuric acid, with a crystal of thymol added as a preservative. The material was tamped to ensure

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intimate contact of the acid with the plant material. The bottle was capped and stored for a week in a refrigerator. During storage the material was mixed and compressed on two occasions. The liquid was compressed out of the bottle and drained into a centrifuge tube. After centrifugation, 15 ml of the supernatant liquid were thoroughly mixed with 23 g of silicic acid (Mallinckrodt, analytical grade, 100 mesh), purified according to Hathway:[10] 100 g of silicic acid were stirred with two 1-litre portions of boiling 2 N-hydrochloric acid for 2 h, the supernatant was decanted and approximately 30% of the finest particles were removed by repeated suspension and decantation. After being washed free from acid with water and then methanol, the adsorbent was dried at 100° for 16 h and stored in a desiccator. The silicic acid/sulphuric acid extract mixture was quickly covered with benzene in order to prevent evaporation from the large surface area of the gel. A glass tube, 3 cm in diameter fitted with a sintered glass disc and a tap at the lower extremity was used for the purification step. The column was prepared by adding first 4 g of silicic acid saturated with 2·5 ml of distilled water and made into a slurry with benzene and the slurry containing the sample was added on top of this. The column was packed with a pressure of 2 lb of nitrogen and the benzene allowed to drain into a separating funnel. Acids were eluted with 450 ml of a 60% v/v solution of butanol in benzene which had been saturated with 0·5 N-sulphuric acid. A few millilitres of water were added to the contents of the separating funnel and the acids were neutralised to cresol red with 0·1 N-sodium hydroxide. The aqueous layer was removed and the solvent was washed twice with water. The combined aqueous fractions were concentrated to a small volume in a rotary evaporator and were taken to dryness over phosphorus pentoxide and pellets of sodium hydroxide in a vacuum desiccator. The salts were dissolved in water and the solution made to 1 ml for application to the analytical column.

Chromatographic analysis

Samples from the two methods of extraction and purification were processed in the same way. The acids were separated on a silica gel column by a method based on the original Isherwood[3] method but improved by the incorporation of many modifications.

Preparation of column

Ten g of dry silica gel, prepared as described above for the purification step, were thoroughly mixed with 6 ml of 0·5 N-sulphuric acid. The adsorbent was made to a thin slurry with benzene and poured into the analytical column (Fig. 1). The column was a 1·3 cm tube i.d. with the upper part fitted with a B29 ground glass joint for connection to the gradient elution apparatus. An aluminium connector joined the column with the lower section. A Teflon stopcock controlled the rate of flow of the eluate and a porous disc supported the column packing. Before the tube was fitted with adsorbent, a filter paper was placed above the disc and a few millilitres of benzene were added. The adsorbent was allowed to settle and the walls of the tube were cleaned free of gel. A pad of filter paper cut to fit snugly into the tube was placed on top of the adsorbent and the column was slightly compressed with a glass rod. In later experiments it has been found convenient to use No. 740–E (Schleicher & Schüll Co.) discs of adsorbent paper 'for the assay of penicillin and other antibacterial substances'. Each disc is of the exact size required and adsorbs evenly a minimum of 0·08 ml of water. A pad of 8 discs is required for the size of sample used. Benzene on top of the filter paper pad was poured out. The samples were added to the column according to the method of Zbinovsky & Burris.[11] The following sequence was followed: 0·1 ml of 10 N-sulphuric acid was added to the surface of the pad, then 0·4

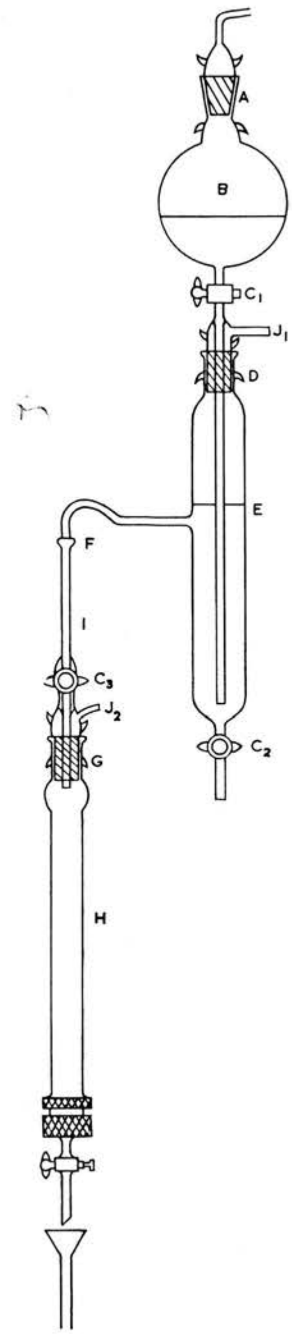

FIG. 1. *Gradient elution apparatus*

A,D B 24 ground-glass joint
B 1 l flask C Teflon stopper
E tube 5 × 32·5 cm, volume 360 ml to line
F ball and socket joint
G B 29 ground glass joint
H 1·3 cm i.d. chromatographic column (adapted)
I capillary tubing J air vents

ml of the sodium (or ammonium) salts from the sample and 0·1 ml of 10 N-sulphuric acid. This was allowed to penetrate into the filter paper and was washed in with two 2-ml portions of benzene. Three ml of benzene were placed above the filter paper and the column was connected with the gradient elution apparatus (Fig. 1) above a fraction collector.

The *gradient elution apparatus* was a modification of the system of continuous gradient elution devised by Donaldson *et al.*[12] and Wall *et al.*[13] It was an all-glass apparatus consisting of a mixing chamber (E) and a reservoir (B). The two parts were connected by a piece of glass tubing extending from the reservoir to the bottom of the mixing chamber. For operation, the mixing chamber was filled to the mark with 360 ml of benzene equilibrated with 0·5 N-sulphuric acid. The air vent (J) on top of the chamber was closed with plastic tubing and screw clip, and the stopcock (C) below the reservoir was closed. Three hundred and sixty ml of a mixture containing 20% of butanol, 80% of benzene equilibrated with 0·5 N-sulphuric acid were poured into the reservoir. When vents were closed, the ball and joint socket (F), firmly secured with a pinch clamp, and stopcocks (C_1 and C_3) were opened, a continuous gradient was produced. It was not necessary to apply external pressure since a flow rate of about 1 ml per minute was attained by gravity. After exhaustion of the contents of the reservoir, the butanol/benzene mixture was removed from the mixing chamber. A second gradient system was started by introducing 360 ml of 20% t-amyl alcohol in chloroform in the mixing chamber and 450 ml of 60% t-amyl alcohol in chloroform in the reservoir. Both solutions had been equilibrated previously with 0·5 N-sulphuric acid.

Fractions of about 4 ml were collected by means of a time-actuated fraction collector. One ml of an ethanolic solution of 0·01% w/v phenolphthalein made slightly alkaline with 0·1 N-sodium hydroxide[7] was added to each fraction which was then titrated with 0·01 N-sodium hydroxide in a 80% v/v ethanolic solution and results were recorded to the nearest 0·01 ml. During titration a fine stream of nitrogen was bubbled through the liquid by means of a capillary tubing. Calculations of acid concentration were done as in the Wiseman & Irvin[9] method.

Characterisation of eluted compounds

The acids were identified by their position of elution from the silica gel column, by paper chromatography in at least two solvents and by specific colour reactions on paper chromatograms. This was judged sufficient for acids which were known to occur in the type of material examined.

^{14}C-measurement in the eluted fractions

In order to measure the radioactivity in the eluted fractions, it was first necessary to ascertain the exact position of elution of the acid concerned. This was done by titrating alternate fractions from the column. A minimum of two fractions from each peak were measured. An accurately measured volume of 0·5 ml of eluate was taken before titration, and mixed with 2 ml of NE 213 liquid scintillator [Nuclear Enterprises (G.B.) Ltd.]. This scintillator was based on xylene, contained naphthalene, activators and 1,4-bis-2-(5-phenyloxazolyl)benzene as spectrum shifter. The fractions had been deoxygenated before sampling by bubbling nitrogen through for 30 sec. The scintillator was similarly deoxygenated. The direct mixing of the eluate with the scintillator was only possible when a benzene/butanol system of elution was used. When the t-amyl alcohol/chloroform solvent was the eluant, 1·0 ml of the fractions was evaporated by keeping the test tubes in a water-bath at 45° and blowing a stream of nitrogen on the surface of the liquid. The acid was then dissolved in 1·0 ml of primene (0·5 M in methanol). A volume of 0·5 ml was blended with 2 ml of NE 213 scintillator. The titre of the fractions was obtained by titrating a 1-ml aliquot of the same fractions.

The counting apparatus was an Ekco Scintillation Counter, Type N664A, coupled to an Ekco Automatic Scaler, Type N530G (Ekco Electronics Ltd.). The apparatus was utilised with the following settings: amplifier gain, 1000; discriminator bias, 15 V; output voltage (H.V.), 1000v. The instrument was calibrated using a uniformly labelled source of ^{14}C-n-hexadecane of known activity.

Results

Recoveries from the chromatographic column

In recovery experiments where 0·4 ml of a synthetic solution containing most of the silage acids and a few plant acids was adsorbed on a silica gel column and eluted by the solvent systems described above, good recoveries were obtained for most acids (Table I). Formic acid and oxalic acid were the most difficult acids to recover quantitatively; nevertheless, recoveries of above 90% were always obtained. Elution of the acids produced sharp peaks. In the beginning of column development, three fractions contained the whole of valeric acid; however, the number of fractions necessary to elute other acids increased considerably, especially toward the end of the elution. It was possible to prepare columns with similar characteristics from one run to the other, but the fractions in which the acids are collected vary from one sample to the other depending on concentration and various other factors including the temperature of the room. Therefore, the fraction numbers presented are shown as an approximation only. The blank values obtained when a column was run under the same conditions but with the sample replaced by 0·5 N-sulphuric acid were low at the beginning of column development, but increased in later stages of the elution.

TABLE I

Performance of chromatographic column
(0·4 ml portions of standard solutions of acid were used)

	Concentration of individual acids in standard solution (mequiv/ml)	Fractions with acids	Average blank value of peak (ml of 0·01 N-NaOH)	Recovery of acids (average of 4 runs) %
(Benzene/butanol eluant) (3·5 ml fractions)				
n-Valeric	0·1	4–6	0·01	98·9
n-Butyric	0·2	8–13	0·01	100·1
Propionic	0·2	15–21	0·01	99·0
Acetic	0·6	27–35	0·02	98·4
Formic	0·1	39–48	0·02	92·5
Succinic	0·2	70–77	0·03	99·3
Lactic	0·6	82–98	0·03	101·1
(Chloroform/t-amyl alcohol eluant) (4·0-ml fractions)				
Oxalic	0·1	106–121	0·04	91·2
Malic	0·6	128–142	0·06	98·3
Citric	0·3	144–158	0·10	98·0

Counting efficiency

Uniformly labelled ^{14}C-n-hexadecane was dissolved in NE 213 scintillator to give a final activity of 2·00 mμc per ml and this standard solution was used for calibrating the instrument.

Final settings of the instrument were adopted where the ratio of (net count rate)^2/(background count rate) was maximised. At these settings, the background count rate was 0·375 counts per second and the counting efficiency of 0·5 ml of the standard mixed with 5 ml of NE 213 scintillator was 66·6%.

The effect of scintillator volume on background count and counting efficiency was investigated with a ^{14}C-n-hexadecane standard solution containing 1 mμc and volumes of 2 ml and 5 ml of NE 213 scintillator. The effect of adding 0·500 ml of effluent from the chromatographic column on counting efficiency and background count is also shown (Table II) for a few selected fractions corresponding to elution peaks of the major organic acids in silage. The smaller volume of scintillator tended to give slightly smaller background count rate and slightly higher efficiency. It was also evident that the solvent from the first stage of the elution with the benzene/butanol gradient could be used over the whole range for determining the radioactivity in organic acids. Attempts were also made to determine directly the radioactivity in malic and citric acid fractions where the solvent was a gradient of t-amyl alcohol/chloroform. The efficiency of counting was decreased by about 95%, on account of quenching due to chloroform. The following procedure was then tested: a 1-ml aliquot[5] of fractions from the malic acid peak was dried in a water-bath at 45° while a stream of nitrogen continuously agitated the surface of the liquid. This was dissolved in 1·0 ml of 0·5 M solution of primene in methanol and 0·5 ml was mixed with 2 ml of NE 213 scintillator and counted. When a standard number of counts was added in 0·5 ml of scintillator, the recovery was quantitative.

More difficulties were experienced with fractions from the citric acid peak. Recoveries were somewhat erratic and varied from 80 to 95% of added counts. The variation could not be completely explained. It may have been due to some residue from the t-amyl alcohol/chloroform solvent, to the higher blank value due to the elution of some sulphuric acid or to some other factor. It was observed that in silage, the broad citric acid peak contained too little of the acid for an accurate titration to be performed on a 1·0-ml fraction. On the other hand, the peaks were very symmetrical and the titration value could be accurately estimated without an actual titration. The whole fractions were therefore used for ^{14}C counting instead of 1·0 ml. The same was done for malic acid fractions and for fractions eluted later than citric acid during column development. Although this was sometimes unnecessary, an internal standard was used for all fractions counted in the course of development with the t-amyl alcohol/chloroform gradient elution system.

TABLE II

Effect of volume of NE 213 scintillator and of increasing butanol concentration in the effluent from a silicic acid column on background count and on counting efficiency

Eluted acid	Fraction No.	Background count per second, Volume of scintillator 2 ml	5 ml	Counting efficiency volume of scintillator 2 ml	5 ml
Valeric	5	0·350	0·363	67·2	66·7
Butyric	11	0·343	0·383	67·6	67·0
Propionic	18	0·341	0·366	67·7	66·3
Acetic	31	0·358	0·377	66·0	66·6
Formic	43	0·349	0·377	66·9	66·7
Succinic	73	0·356	0·365	66·5	66·0
Lactic	89	0·361	0·375	67·9	65·2

Efficiency of extraction and purification procedures

One of the main difficulties in the determination of organic acids from plant tissues is their extraction. The purification of the extract is also cumbersome. The usual method of extraction with 80% ethanol,[7,8] improved by following it with a water extraction,[14] was tested against an acid-extraction method. With the alcohol followed by water extraction, purification was affected by passage through ion-exchange resins, while the acid extract was purified on a silica gel column. Red clover (*Trifolium pratense*) was used as the test material. Details of the extraction and purification procedures have been given above.

Results are shown in Table III and a histogram of the distribution of acids obtained is shown in Fig. 2. Using a combination of silica gel chromatography and paper chromatography, at least 19 acids were detected and the most abundant ones have been identified. The only major acid which was not identified is the one under peak No. 16. Actually this acid accounted for more than 37% of the acids titrated. The next acid in order of magnitude was malic acid while malonic, fumaric, citric and acetic acids were important components and oxalic, glycollic and lactic acid also appeared. Traces of other acids (less than 0·4 mequiv./100 g dry matter) were also detected but not identified or measured quantitatively.

TABLE III

Organic acids of red clover (mequiv/100 g dry matter) determined by silica gel chromatography, after two different methods of extraction

Peak No.	*Method of extraction* Dilute Acid	Alcohol followed by water	Identification, specific reagent used on paper chromatograms and reference
	mequiv./ 100 g	mequiv./ 100 g	
1	trace	0·0	Unidentified
2	trace	trace	Unidentified
3	trace	trace	Unidentified
4	trace	0·0	Unidentified
5	trace	0·0	Unidentified
6	5·4	1·3	Acetic acid
7	7·2	2·3	Fumaric acid, absorption in ultra-violet potassium ferrocyanide, then ferric ammonium sulphate[15]
8	trace	0·0	Unidentified
9	0·8	1·0	Succinic acid, potassium ferrocyanide, then ferric ammonium sulphate[15]
10	11·4	11·1	Malonic acid, trace of lactic acid, diazotised *p*-nitroaniline,[13] ammoniacal silver nitrate[16]
11	1·4	0·9	Oxalic acid, potassium ferrocyanide, then ferric ammonium sulphate[15]
12	trace	trace	Glycollic acid + 2 acids, ammoniacal silver nitrate[16]
13	trace	0·0	Unidentified
14	20·5	21·0	Malic acid, R_F-values in 3 solvents
15	7·1	5·7	Citric acid, aroyl-glycine reagent[17]
16	34·0	34·8	Unidentified; positive to Cartwright & Roberts reagent,[18] positive to ammoniacal silver nitrate[16]
Total anions		153·8	
Total determined	89·6	78·8	
Organic acids extracted	100·5		

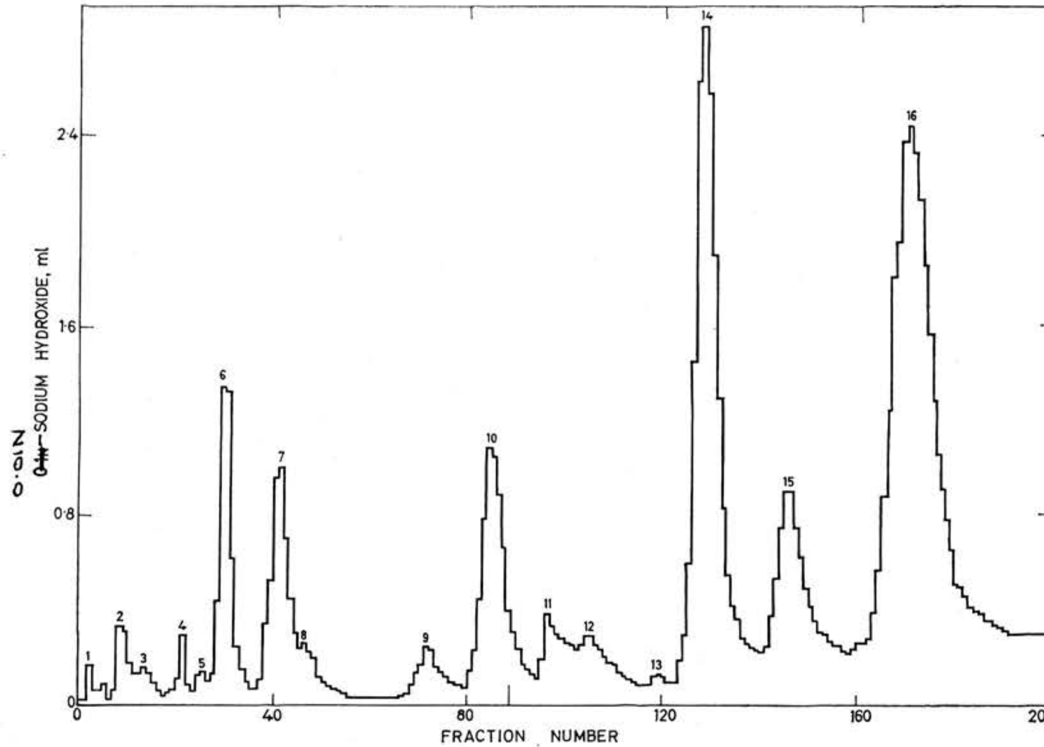

FIG. 2. *Histogram of distribution of acids from red clover using the acid extraction procedure* (for key to peak numbers see Table III)

Elution: up to fraction 88, gradient of benzene → 20% butanol in benzene beyond fraction 88, gradient of 20% t-amyl alcohol in chloroform → 60% t-amyl alcohol in chloroform

The two methods of extraction and purification have yielded similar results for the three major acids, but results were not similar for citric, oxalic and fumaric acids. The acid extraction showed a superiority for the determination of those acids. Acetic acid was also present in larger quantities in the acid-extracted samples. Succinic acid was in larger quantities in the alcohol followed by water extraction and purification on ion-exchange resins.

Discussion

Methods of extraction and purification

In the separation of organic acids from plant materials, the most frequently employed method has been the extraction with 70 or 80% ethanol, especially when a preliminary fractionation of the extract into organic acids, amino-acids and neutral materials was desired.[1, 2, 7, 8] The use of inorganic acid is made difficult by the necessity of removing the swamping acid which would interfere with subsequent chromatography. However, Fauconneau,[14] fractionating his extracts on Dowex-1 anion exchangers, has found it possible to extract the acids from plant material with dilute hydrochloric or perchloric acid, removing the inorganic acid by precipitation as the potassium perchlorate at 0° or taking the extract to dryness repeatedly. The removal of perchloric acid by precipitation was not satisfactory for silica gel chromatography since the traces of perchloric acid left were eluted with malic acid and subsequent fractions. Hydrochloric acid volatilisation was not satisfactory either because of the resulting loss of volatile acids. Fauconneau[14] has also shown that an aqueous extraction after the alcoholic extraction was a more efficient extraction method than with alcohol alone. The method of extraction with dilute sulphuric acid presented above has been evolved from Isherwood's[3] extraction method for organic acids in fruits. A similar method has been used for silage acids by Wiseman & Irvin[9] and by Wilson & Tilley.[19] For ease of concentration it was necessary to keep the volume of dilute sulphuric acid to a minimum. On the other hand, too concentrated a sulphuric acid solution might result in the production of artifacts. Sulphuric acid at a concentration of 0·3 N has been used in the present study since there is only a slight excess after salt splitting in the plant material. For use in plants containing less organic acids than clover, such as the grasses, a 0·2 N solution might be sufficient. In silage, 0·6 N-sulphuric acid would be required to reduce the pH to 2·0.

In confirmation of the work of Fauconneau[14] and Roux & Lesaint,[20] we have found that ethanol and water extraction do not extract all the organic acids from plant material. The insoluble calcium citrate and calcium oxalate were not completely extracted by ethanol even when followed by aqueous extractions. This would suggest that figures reported in the literature for those two acids are frequently an underestimate of the true value. Many acids, which were present in trace amounts with the dilute acid extraction, were completely absent in the alcohol extract. It is likely that they were retained on the ion exchangers. Similar retention of aromatic acids has been noted by Wall *et al.*[13] Fumaric acid was probably also retained on the ion exchangers.[21] It is difficult to explain why acetic acid was present in smaller quantities in the alcohol + water extracts. Some loss was probably possible through volatilisation, even if all precautions were taken to avoid that.

It can therefore be concluded that the method of extracting acids from plant material with 0·3 N-sulphuric acid was clearly satisfactory and can replace with advantage the usual alcohol extraction. The use of silica gel for purification had an added advantage. It separated the organic acids from inorganic anions, which, when present, are partly eluted with citric acid and other acids eluted after it. In our sample, the difference between the total anions as measured by titration of an aliquot of the eluate from the Amberlite IR–120 resin and the organic anions eluted from the silica gel column was 53·3 mequiv./100 g. (Table III). This would indicate that about one-third of the anions were inorganic anions.

It was also found that the purification of the extract on the silica gel column was much easier and more convenient than on ion-exchange resins. The use of benzene/butanol as the eluant instead of the usual chloroform/butanol[3, 7] was found more convenient, as removal of the water layer and washings after titration can be done without removal of the solvent layer.

Chromatographic separation on silica gel

The technique employed was very similar to that of Wall *et al.*[13] The main difference was that a benzene-based eluant was used for the first phase of the separation. The reason for this was that benzene does separate succinic acid from lactic acid[22] while chloroform does not, especially if lactic acid is in large quantities as is the case for silage. In addition, with benzene it is possible to separate valeric from butyric acid. It is even possible partly to separate caproic from valeric acid. One advantage of the apparatus utilised is its extreme versatility. The eluant can be changed at will almost at any stage of the elution. If desired, it is possible to start elution with pure benzene for separating propionic from butyric acid. As shown in Table I, there is little separation between those two acids and, if butyric is in large quantities, it might overlap the first propionic acid fractions. In such cases it would also be advantageous to collect smaller fractions of 2 ml for example. With the elution as described, there was no separation between lactic and malonic acids. If such a separation is desired (as in silage made from legumes) it is possible to change from the benzene/butanol gradient elution system to a new gradient immediately after succinic acid is eluted, after about 270–280 ml of solvent have passed through the column. The gradient elution system should
5, consist of 360 ml of ~~10~~% t-amyl alcohol in chloroform in the
15, mixing chamber and ~~20~~% t-amyl alcohol in chloroform in the reservoir. Elution can be continued after elution of lactic acid with the usual 60% t-amyl alcohol in chloroform.

The continuous gradient-elution apparatus has the further advantage that it can be left unattended for long periods of time. If the reservoir empties after elution with benzene/butanol, the column does not dry immediately.

Scintillation counting

As shown in Table II, the benzene/butanol solvent system was satisfactory for direct addition to the scintillator. When counting of fractions was performed on samples containing radioactivity, and an internal standard was added to verify efficiency, quantitative recoveries were obtained. This has also been verified with fractions from the t-amyl alcohol/chloroform system. With the first solvent system it was also dossible to use the technique of elution coincidence,[23] which provided a proof of the lack of contamination of the acid separated. The efficiency obtained with benzene or with benzene/butanol for counting were similar to those reported by Stitch,[24] but were not as high as those reported by Brown & Badman.[25] Primene has been found useful for dissolving non-volatile carboxylic acids and satisfactory efficiencies have been obtained. This finding agrees with the results of Radin & Fried.[26]

Organic acids in red clover

The presence of malic, malonic, oxalic, glycollic, lactic, fumaric, succinic and acetic acids in red clover has been established. The presence of most of these acids in other forage plants had been reported previously by Fauconneau,[27] by Hulme & Richardson,[28] and by others, but to our knowledge, it is the first time they are reported in red clover. In addition to these acids, another acid was found, which is the major acid of red clover. The acid is eluted in approximately the same position as shikimic and citric acids but does not have the same R_F value on paper chromatograms. Its response to the ammoniacal silver nitrate test[13] indicates the presence of a glycol group in its constitution. Work is continuing on its identification and this will be reported in a later publication.* In addition to the above, not less than 9 unidentified acids have been found, but they were not present in sufficient quantity for characterisation. Quinic acid has not been determined in the present work. It would not be detected in the present elution system as it would be masked by the high blank value. A more suitable method of separation for quinic acid would be chromatography on a strongly basic anion-exchanger in the acetate form or formate form.[27, 28] The presence of quinic acid has been reported in most agricultural plants.[27, 28] Aconitic acid was not found in the present samples of red clover, although both the cis- and trans-isomers were found in other samples of red clover.

Rapid method of detection

The method as described is not quite rapid enough for routine use. However, for fermentation studies where the interest might be only in the volatile acids, lactic and succinic acids, the method may be shortened considerably, provided the acid concentration of the extract is high enough. The purification and concentration steps are omitted. Two ml of the properly acidified extract are mixed with 3 g of the silica gel and the resulting powder is added to the top of the column[19] over a filter paper. It is then slurried with a small quantity of benzene. A second filter paper is placed on top of the sample and the column is developed as before, omitting the t-amyl alcohol/chloroform solvent. The titration procedure is also shortened by adding to each tube before collection 1 ml of an alcoholic cresol red solution made 0·0005N with sodium hydroxide. The appropriate fractions are pooled before titration. Under such conditions four columns can be developed simultaneously in one day using a four-row, 240-tube fraction collector. Columns can also be re-used by scraping off the adsorbent used to add the sample.

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ADDENDUM

Since the manuscript of the above paper was submitted, a further study has been made of the unidentified acid present in red and white clovers and reported therein, and tentatively identified as glyceric acid by Playne & McDonald.[1] Samples of the acid from both species were purified by paper chromatography and the crystalline calcium salts were prepared. Both samples had the same melting point (138°) and infra-red spectra identical with that of an authentic sample of calcium D-glycerate. Although as far as the authors are aware, this acid has not been found previously in clovers, its presence in cress seedlings,[2] tobacco leaves,[3] broad bean leaves[4] and field bean leaves[5] has been reported. (The authors would like to acknowledge the help of Dr. A. W. MacGregor in the preparation of the pure salt and its identification.)

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THE BUFFERING CONSTITUENTS OF HERBAGE AND OF SILAGE

By M. J. PLAYNE* and P. McDONALD

The buffering capacity values of several herbage species and of silage made from this herbage, and the contributions of plant constituents to this buffering, between pH 4 and 6, were determined, and found to vary with the herbage species; values in the silages were normally two to three times greater than those in the plant materials. The anion fraction of the plant materials accounted for 68–80% of the total buffering capacity, and for 73–88% in the silages. Buffering caused by plant proteins was estimated to be 10–20% of the total buffering capacity.

The buffering capacity of wilted red clover (*Trifolium pratense*) was 18% lower than that of fresh red clover, and the total organic acid content of wilted clover was also lower than that in fresh clover.

The organic acids were responsible for most of the buffering effect in herbages and silages. In Italian ryegrass (*Lolium multiflorum*), the main buffers were malate and citrate, but, in red clover, glycerate and malate were the main buffers. The clovers studied contained a high level of glycerate (4% of dry matter). During ensilage, malate, citrate and glycerate were extensively broken down. The increased buffering capacity during ensilage was caused mainly by the formation of lactates and acetates.

Introduction

Several workers[1–6] have determined buffering capacity values on aqueous macerates of a number of herbages and silages. Smith[3] further divided an aqueous plant extract by a dialysis and ashing procedure, and concluded that inorganic ions largely caused the buffering of lucerne. However, he apparently ignored the possible rôle of the plant organic acids, and used lactic acid as the titrant. Lactic acid, itself, forms a buffer system in the presence of inorganic cations. Yet, Dunne[7] has suggested that the plant organic acids and their salts form the most important buffer systems in the plant in the acid region ($pH < 6$). The pH range 6–4 is the most important range in these studies, since most plant materials have pH values about 6, and good-quality silages, made from unwilted material, usually have pH values about 4.

It has been suggested[2] that liberated proteins are the major buffering substances in the acid region, despite contrary evidence.[8] It has been shown since that the protein content of a species did not directly control the buffering capacity,[4, 5] and that the increase in buffering capacity during ensilage was not apparently related to the increase in the products of protein breakdown.[5]

Neutralising power and buffering capacity have not been clearly differentiated in previous work. Fresh plant material contains buffering substances, but during ensilage, neutralising substances are released as well as additional buffering systems being formed.

In this study, the buffering constituents of fresh herbage and of silage, made in test-tube silos, were examined for several pasture species. Although it is understood that conditions in small tubes may not exactly simulate those of large farm silos where free drainage occurs, previous studies have shown that, in both test-tube and large metal silos (1000 kg capacity), chemical and bacteriological changes follow the same pattern.

Plant and silage extracts were divided into fractions by ion-exchange techniques, and the anion fraction was further separated into the individual organic acids. An attempt was made to assess the buffering effect of plant proteins.

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Experimental

All analyses were carried out on fresh material.

Silos

Fifty g capacity laboratory silos were used. These test-tube silos have been described previously.[9]

Dry matter

Fresh plant material (300 g) was dried in a forced-draught oven at 75° for 16 h (or until constant weight). Normally, the dry matter content of silage was not determined, and the results of silage analyses were expressed on the basis of ensiled plant dry matter. Fresh silage samples taken represented a known amount of plant dry matter.

pH value and buffering capacity

Fresh material (10 g or 20 g, depending on the type of plant material and on the dry matter content) was macerated with 250 ml of distilled water. The pH of the macerate was recorded, and the buffering capacity was measured by electrometric titration using a Cambridge pH meter. The macerate was titrated first to pH 3 with 0·1 N-hydrochloric acid in order to release bicarbonate as carbon dioxide, and then was titrated to pH 6 with 0·1 N-sodium hydroxide. Buffering capacity was expressed as mequiv. of alkali required to change the pH from 4 to 6 per 100 g of dry matter, after correction for the titration value of a 250-ml water blank.

Fractionation of the fresh material

Fresh material (20 g) was stored in 80% (v/v) ethanol at −15° until required for analysis. It was then extracted successively with 80% (v/v) ethanol, with cold water, and with hot water (60°) by repeated maceration, filtration and washing.

The pale fibrous residue which remained was taken up in 250 ml of water, and the buffering capacity of this suspension was measured as described above.

The total filtrate, from the successive extractions, was then titrated to pH9 with sodium hydroxide, and concentrated in a rotary film evaporator under reduced pressure at 40° in order to remove ethanol and to reduce the volume. The concentrated aqueous extract was washed with chloroform (ethanol-free) to remove lipids and other interfering materials. The aqueous extract (~100 ml) was passed through a cation- and then through an anion-exchange resin column. The columns (both 9 mm × 200 mm) each held 12 ml of wet resin. The resins used were Amberlite IR-120 (H^+ form, 10–20 mesh), which adsorbed the amino-acids and the inorganic cations, and Amberlite IR-45 (weakly basic, OH^- form, 10–20 mesh), which adsorbed the anions. The resins were prepared, regenerated and washed in the usual manner.

(a) *Neutral fraction.*—The final eluate and washings from the columns contained the neutral substances such as the sugars. This fraction was acidified to pH3 with 0·1 N-hydrochloric acid, made up to 250 ml, and the buffering capacity was measured.

(b) *Amino-acid fraction.*—The amino-acids were eluted from the cation-exchange resin with five bed-volumes of 4 N-aqueous ammonia followed by five bed-volumes of water. After addition of 10 ml of 0·1 N-sodium hydroxide, this eluate was evaporated to remove ammonia, and then was made up to 250 ml with water, acidified to pH3 to remove bicarbonate, and finally titrated to determine buffering capacity.

(c) *Inorganic cation fraction.*—After removal of amino-acids from the cation-exchange resin by elution with ammonia, the inorganic cations were eluted with 1 N-hydrochloric acid, and the buffering capacity of this fraction was determined. Since, preliminary studies showed that the buffering capacity of this fraction was negligible, it was not normally determined.

(d) *Anion fraction.*—The organic anions and other anions, such as phosphates, sulphates, nitrates and chlorides, were eluted from the anion-exchange resin with 1 N-hydrochloric acid. The eluate was diluted to 250 ml with water and the buffering capacity of this solution was determined.

Organic acids

(a) *Extraction.*—The acids were extracted by the method described above, except that after extraction with hot water, 10 ml of wet cation-exchange resin (Amberlite IR-120, H^+ form, 10–20 mesh) were added and macerated for 5 min. with the residue, and then the suspension was stirred for a further 10 min. This technique served to free organic acids existing as insoluble salts in the residue. The residue and resin mixture was discarded after washing and filtration. The acid filtrate was added to the combined filtrate (ethanol + cold water + hot water), and reduced the pH of the combined filtrate to about pH3. This resulted in the precipitation of most of the colloidal material, including the proteins. The combined filtrate was then re-filtered and titrated to pH9 with sodium hydroxide before concentration to a small volume to remove ethanol.

The filtrate was passed through cation- and anion-exchange resins, and the organic acids were eluted from the anion-exchange column with 0·5 N-sodium hydroxide. The free acids were formed by passing this eluate through cation-exchange resin. The solution containing the free acids was titrated immediately to pH9 with 0·1 N-sodium hydroxide to avoid loss of volatile acids. Total titratable acidity could be calculated from this titration. The solution was then evaporated to dryness in a rotary film evaporator under reduced pressure at 40°, and in a vacuum desiccator over phosphoric oxide and sodium hydroxide pellets.

(b) *Separation.*—The acids were separated by silica gel column-chromatography using the technique of Lessard & McDonald.[10, 11]

Paper chromatography[12, 13] and specific spot tests[14] were used to help to identify the acids found.

Results

Buffering capacity of authentic samples

(a) *Organic acids.*—Solutions of the individual organic acids were titrated to pH9, and the titre between pH4 and 6 was recorded. In this way, the buffering capacity of an organic acid between pH4 and 6 could be calculated. This value varied, of course, with the dissociation constants of the acids. The results obtained are shown in Table I.

TABLE I

Contributions to the buffering capacity (pH4–6) of individual organic acids

(The ratio is defined as titre pH4 to 6/total neutralisation titre)

Acid	Ratio	Acid	Ratio
Butyric	0·84	Fumaric	0·40
Acetic	0·80	Formic	0·37
Succinic	0·69	Malonic	0·35
Malic	0·53	Lactic	0·33
Citric	0·46	Quinic	0·28
Glyceric	0·40		

(b) *Phosphates.*—The buffering capacity of disodium hydrogen phosphate was examined. The solution prepared contained 0·3 g of phosphorus, and a titre of 1·35 mequiv. of hydrochloric acid was required between pH4 and 6. Temperate grasses contain, on average, about 0·3 g of total phosphorus/100 g of dry matter. Thus, the contribution of phosphates to the buffering capacity (pH4–6) would not normally exceed 1·35 mequiv./100 g of dry matter. Total buffering capacity of grasses is normally 25–40 mequiv./100 g of dry matter. Hence, the contribution of phosphates is small. In addition, only about 50% of total phosphorus in the plant exists as titratable inorganic orthophosphate.[15]

(c) *Bicarbonates.*—When grass macerates, of initial pH about 6, were titrated to pH3, then aerated with carbon dioxide-free air, and finally titrated back to pH6, the buffering capacity value from pH6 to 4 was not greater than that from pH4 to 6. This finding indicates that bicarbonate does not form an important buffer system in fresh grass. Any bicarbonate present in the fresh herbage before titration would have been released as carbon dioxide gas when the macerate was acidified to pH3 and aerated.

A sample of sodium bicarbonate (10 mequiv.), dissolved in 250 ml of water, was titrated to pH3 from its original pH of 8·24. The titre required between pH6 and 4 was only 23·1% of the total titre to pH3. Thus, a considerable amount of bicarbonate would have to be present to cause much buffering between pH6 and 4. However, a bicarbonate buffer system may form in the early stages of ensilage when the pH of the mass is still about pH6, and when large quantities of carbon dioxide are being evolved as a result of respiration and decarboxylation.

Comparison of hydrochloric and lactic acids as titrants

These acids were compared because of the number of studies made using these titrants.[1–6] The titration of sodium lactate by hydrochloric acid is compared in Table II with the titration of sodium lactate by lactic acid.

Lactic acid (1·58 mequiv.; 0·142 g) in 170 ml of water was titrated to pH9 with 0·1 N-sodium hydroxide (stage 1), then titrated to pH4 with either 0·1 N-hydrochloric acid or 0·1 N-lactic acid (stage 2). Samples were then titrated to pH6 with 0·1 N-sodium hydroxide (stage 3), and finally back again to pH4 with hydrochloric acid or lactic acid (stage 4).

TABLE II

Comparison of hydrochloric acid and lactic acid as titrants*

Stage	Titrant	Sample 1	2	3	4
1	NaOH	0·52	0·52	0·52	0·53
2	or HCl	0·52	0·48	—	—
	or Lactic acid	—	—	0·90	0·90
3	NaOH	0·50	0·51	0·76	0·82
4	or HCl	0·48	—	0·79	—
	or Lactic acid	—	0·76	—	1·34

*Results are expressed as mequiv. of acid or alkali required between pH4 and 6

Titration with lactic acid caused an increase in buffering capacity, between pH4 and 6, of the solution; but the sole use of hydrochloric acid/sodium hydroxide did not cause any marked change in the buffering capacity (Table II).

Hydrochloric acid was preferred to lactic acid in subsequent buffer determinations, because it was considered more desirable to titrate with a substance which did not buffer between pH6 and 4. Lactic acid, however, could be used as it is possible to calculate the increased buffering caused by the addition of lactic acid during a titration (see also, Table I).

Experiment 1—The buffering capacity of ion-exchange fractions of herbage and silage

A mixture of broad red clover (*Trifolium pratense*) and Italian ryegrass (*Lolium multiflorum*), in a 9 : 1 dry weight ratio, was ensiled in 1000-kg capacity experimental silos for 200 days.[16] The buffering capacity values of the several ion-exchange fractions are given in Table III.

TABLE III

Buffering capacity values of ion-exchange fractions of a broad red clover/ryegrass mixture

(values given as mequiv./100 g of dry matter)

	Plant material		Silage	
Total buffering capacity	69·9		121·8	
Buffering capacity of fractions:				
Anion	47·8	(68·4)*	99·3	(81·5)*
Neutral	0·7	(1·0)	nil	
Cation	3·5	(5·0)	4·5	(3·7)
Extracted residue	10·9	(15·6)	15·0	(12·3)
Sum of fractions	62·9	(90·0)	118·8	(97·5)

*Figures in parenthesis are the values expressed as per cent of total buffering capacity

Since the anion fraction made such an important contribution to the buffering capacity between pH4 and 6, a study of the organic acids, which are contained in this fraction, was made. In Experiments 2 and 3, the quantities of the individual organic acids were determined, and the buffering effect of these acids between pH6 and 4 were calculated from Table I.

Experiment 2—Buffering studies on Italian ryegrass and 31-day Italian ryegrass silage

Samples of Italian ryegrass (S22) were harvested at the flowering stage on October 18th, 1963. The composition of this grass was: dry matter, 23·3%; total nitrogen in the dry matter 1·70%; water soluble carbohydrates in the dry matter 20·3%. The grass was ensiled in twelve 50-g capacity test-tube silos. Three replicate tubes at each date were opened at 3, 8, 15 and 31 days after ensiling. Total buffering capacity and pH values of the silages are shown in Table IV, together with the mean buffering capacity values of ion-exchange fractions of the plant and silage extracts.

TABLE IV

Changes in pH, total buffering capacity, and buffering capacity of ion-exchange fractions of Italian ryegrass during ensilage*

	Period of ensilage (days) 0	3	8	15	31
pH†	5·89	4·39	4·27	4·19	4·31
Total buffering capacity (macerate)**	31·0	62·7	75·1	86·0	100·0
Buffering capacity of fractions:					
Anion	21·2	49·6	62·3	75·9	80·0
Amino-acid	2·3	5·3	7·2	4·3	9·2
Extracted residue	7·1	8·2	7·2	6·5	5·9
Sum of fractions	30·6	63·1	76·7	86·7	95·1

*Buffering capacities are expressed as mequiv./100 g dry matter
†Mean values are all significantly different ($P < 0·05$)
**Mean values are all significantly different ($P < 0·01$)

The organic acid composition of the fresh grass and of the 31-day silage are shown in Table V. The analytical method used cannot detect quinic acid which is usually present in Italian ryegrass.[17] Acids such as butyric, propionic, aconitic, pyrrolidone carboxylic, shikimic and tartaric acids were not present.

TABLE V

Organic acid composition of Italian ryegrass, and red clover, and of their silages*

Acid	Italian ryegrass Fresh grass	Italian ryegrass 31-day silage	Red clover Fresh clover	Red clover 8-day silage
Valeric	nil	nil	nil	0·20
Acetic	nil	51·35	1·75	38·04
Formic	nil	4·15 (Formic + Fumaric)	nil	trace
Fumaric	2·63		8·05	4·30
Succinic	1·00	7·23	0·58	13·46
Malonic	3·01	3·00	15·13	15·00
Lactic	nil	110·26	trace	133·50
Oxalic	nil	nil	0·75	1·84
Glycollic	2·21	3·06	2·28	1·28
Malic	18·65	0·95	26·63	0·93
Citric	10·73	1·25	3·92	nil
Glyceric	nil	nil	37·59	9·99
Total	38·23	181·25	96·68	218·54

*Results expressed as mequiv./100 g dry matter (quinic acid was not determined)

Malic and citric acids were the major acids of fresh ryegrass and formed 76·9% of the total acids detected. During ensilage, almost complete breakdown of these two acids occurred, which confirms previous work.[18] Large quantities of lactic and acetic acids were formed, and the quantity of succinic acid increased markedly. Later work[19] using aseptic grass showed that at least part of this increase in succinic acid arose from plant enzymic processes.

The buffering effect, between pH4 and 6, of the various acids differed somewhat from the absolute amounts present (see Table I). The buffering capacity, pH6 to 4, of the total acids detected was 18·41 and 87·08 mequiv./100 g of dry matter for the grass and the silage respectively. In fact, acetate (40·87 mequiv.) was a more important buffer between pH4 and 6 in the silage than was lactate (36·39 mequiv.) even though twice as much lactate was present.

Experiment 3—Buffering studies on clover and clover silage

Broad red clover samples were cut at an early flowering stage on 24th June 1963. In addition to a study of the fresh herbage, the effect of wilting on the buffering constituents of broad red clover was also examined. Fresh clover (1000 g) was wilted for 48 h at room temperature from 14·25% to 31·8% dry matter. There was no loss of the dry matter originally present during wilting.

Silage was made in 50-g capacity test tube silos from:

(*a*) fresh clover, minced in an electric mincer;
(*b*) fresh clover, chopped to about 1 in. in length;
(*c*) wilted clover, chopped to about 1 in. in length.

All materials were separately ensiled for 8 days at 30°.

The mean pH and buffering capacity values of the plant materials and the silages from three replications are given in Table VI.

TABLE VI

pH and buffering capacity values of fresh and wilted red clover samples and their silages*

	Plant materials			8-day silages		
	Minced	Chopped	Wilted	Minced	Chopped	Wilted
Dry matter, %	15·0	13·8	31·8	—	—	—
pH value	5·95	5·73	5·80	4·02	4·35	5·03
Buffering capacity (pH4 to 6)	57·8	61·7	49·1	142·2	147·1	76·3
Buffering effect of total organic acids found (calculated from Tables I and V)	42·5	—	8·1	96·5	—	12·4

*Buffering values are given as mequiv./100 g of dry matter

The buffering capacity values of the wilted materials were significantly lower ($P < 0.05$) than those of the minced and chopped materials which did not differ significantly ($P = 0.05$).

The buffering capacity values of the anion fraction were 66–68% of the total buffering capacity values in fresh and wilted red clover, and was 73–79% of the total buffering capacity in all the silages.

The buffering capacity of the wilted clover was 18% lower than that of the macerated herbage, and the increase in the buffering capacity of the wilted material during ensilage was small compared to that in the unwilted materials. This was because smaller quantities of acetate and lactate were formed and this difference is also reflected in the higher pH value of the silage.

The organic acids of fresh minced clover and its silage are shown in Table V.

During wilting, a quantitative decrease occurred in all the acids detected. This loss accounts for the lower buffering capacity of wilted clover plants.

The major acids found in fresh minced clover were glyceric, malic and malonic acids, which formed 82% of the acids detected. In the silage, lactic and acetic acids were formed and accounted for 78·5% of the acids present. The quantity of succinic acid increased markedly, but the quantities of malic, glyceric and citric acids decreased considerably during ensilage. Lessard & McDonald[10, 11] had previously reported the existence in red clover of a quantitatively important unidentified acid. This acid was tentatively identified as glyceric acid by the naphthoresorcinol test,[14] and by paper chromatographic studies when the authentic acid was compared with the unidentified acid using three solvent systems.[12, 13] The acid was also the major acid of white clover (*Trifolium repens*) and formed 43·5% of the acids present.

Buffering properties of proteins

Fresh plant material (50 g) was extracted with a sodium borate buffer solution (M/40; pH9·2) in order to extract soluble protein as well as plant constituents more readily soluble in aqueous solutions. The buffering capacity, between pH6 and 4, of the extract containing protein was determined. The proteinaceous matter was coagulated at pH3·5, and removed from the extract. The extract was then back-titrated to pH6, and the difference in buffering capacities, between pH4 and 6, of the extract with and without protein was estimated as the buffering capacity of the protein. In order to estimate the proportion of protein extracted from the plant material by borate buffer extraction, total nitrogen determinations were made on the original plant material, on the extracted residue, and on the proteinaceous precipitate. This method was not satisfactory mainly because it was difficult to estimate accurately the proportion of protein extracted and because of the interference caused by the bicarbonate-carbonic acid buffer system which may be operating.

The estimated buffering capacity, between pH4 and 6, of protein was no more than 11 mequiv./100 g of dry matter in both Italian ryegrass and red clover. In Italian ryegrass, buffering caused by protein was estimated at 24·2% of the total buffering capacity; and in red clover, at 19·5% of the total buffering capacity. A considerably lower value (8·0% of total buffering capacity) was obtained for a sample of cocksfoot (*Dactylis glomerata*).

Other studies

The buffering capacity and pH value of a number of herbage samples and silages were determined. These, together with the summarised results of samples previously described, are given in Table VII.

TABLE VII

Total buffering capacity and pH values of a number of herbages and silages

(results as mequiv./100 g of dry matter)

Species*	Plant Buffering capacity	pH	Silage Buffering capacity	pH
1. Cocksfoot	24·7	—	—	—
2. Cocksfoot	25·3	—	—	—
3. Italian ryegrass	58·9	5·88	125·0	4·14
4. Italian ryegrass	44·6	—	—	—
5. Italian ryegrass (low N)†	31·0	5·89	100·0	4·31
6. Italian ryegrass (high N)†	38·6	6·16	130·6	4·37
7. Perennial ryegrass	38·6	6·06	89·9	5·81
8. Perennial ryegrass	42·8	5·90	82·2	5·36
9. Red clover (minced)	57·8	5·95	142·2	4·02
10. Red clover (chopped)	61·7	5·73	147·1	4·35
11. Red clover (wilted)	49·1	5·80	76·3	5·03
12. White clover	51·2	—	—	—

*All herbages were at the flowering stage, except samples 3, 7 and 12

†Reference in parenthesis refers to fertiliser treatment

Discussion

The total buffering capacity (pH4–6) of the herbages studied ranged from 31 to 70 mequiv., with the higher values being obtained for clover or clover-rich mixtures. Other studies[19] have confirmed that clovers have approximately twice the buffering capacity of the ryegrass and this is clearly an important factor associated with the difficulties encountered in the ensilage of leguminous crops.

The advantages of wilting herbage prior to ensiling have been stressed by many workers and it is clear from the results presented, that wilting has a beneficial effect in reducing the buffering capacity. This conclusion supports the findings of Smith[3] who worked with fresh and wilted lucerne. The decrease in buffering capacity probably results from the reduction in organic acid content during wilting.

During ensilage, buffering capacity increases markedly as is shown in Table VII. This increase, which can occur rapidly and may double within three days of ensilage, can be attributed to the formation of lactates and acetates. A similar finding was noted by Greenhill.[6] Most of the buffering properties of herbage can be attributed to the anion fraction which includes organic acid salts, orthophosphates, sulphates, nitrates and chlorides. Between pH4 and 6, however, only the organic acid anions and the orthophosphates would buffer and the effect of the orthophosphates in this range would be small. In these studies the anion fraction accounted for about 68% of the total buffering capacity of the herbage samples examined and about 80% of the silages. The results of unpublished data for a number of herbage and silage samples indicate that the contribution of the anion fraction to the total buffering capacity may range from 68 to 80% for fresh herbages and from 73 to 88% for silages.

The buffering components of the residue after successive extraction with ethanol, cold and hot water are not known but may include residual salts of citric and oxalic acids. It has been shown that the calcium salts of these acids are not completely extracted with hot water unless the free acids are first formed.[20] Reference has already been made to this in an earlier paper.[11] The residue also includes cellulose, hemicelluloses, lignin, some proteins and pectic substances.

Considerable breakdown of plant organic acids occurs during ensilage. These acids normally exist as salts in fresh plant material (pH6) and thus their breakdown would result in an excess of cations. Consequently, although the buffering capacity of the material would be lowered by the breakdown of such acids, the excess cations would have to be neutralised by fermentation acids, such as lactic or acetic resulting in little change in total buffering capacity.

Although the experimental technique used to determine the buffering effect of proteins was not considered entirely satisfactory, it is apparent from these studies that proteins make only a small contribution to the buffering capacity of herbage between pH4 and 6 which confirms earlier observations.[4, 5] If it is assumed that only the side-chain carboxyl groups of glutamic and aspartic acids are responsible for the buffering effect of proteins between pH4 and 6, then the hypothetical buffering value for grass proteins is about 6–11 mequiv./100 g of dry matter which is in agreement with the values found in these studies.

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THE EFFECT OF TEMPERATURE ON ENSILAGE

By P. McDONALD, A. R. HENDERSON and R. WHITTENBURY

An experiment was carried out to study the chemical and bacteriological changes during the ensilage of fresh and partially wilted grass at two different temperature levels. In grass of low dry-matter content (15·2%) the effect of increasing the temperature to 42° resulted in a clostridial type fermentation, whereas in similar material kept at 20° a lactic acid fermentation occurred. Differences between the two wilted silages were less marked, although the wilted material at low temperature contained more lactic acid than the high-temperature silage. Results of digestibility trials with sheep did not show any marked variation between treatments, although the digestible crude protein contents were highest in the wet silages. The importance of maintaining low temperatures and of wilting crops prior to ensiling are discussed.

Introduction

In silage making, temperature has always been regarded as a major factor influencing the fermentation process. Towards the end of last century, a temperature of at least 50° was advocated[1] and this was achieved in practice by delaying consolidation until the mass had heated up. The disadvantages and losses associated with this 'warm fermentation process' have been summarised by Watson & Nash,[2] and Murdoch.[3] With the introduction of the flail harvester, consolidation of ensiled herbage has become easier and consequently temperatures lower than 25° are frequently achieved in practice. In spite of the advantages of the flail harvester, there is still a considerable amount of silage made in which the temperature of the mass is within the range 30–50° and this is particularly the case when wilted or partially wilted crops are ensiled.

Studies on the effects of temperature fall into two main categories—those in which the temperature is influenced in silos by varying the degree of aeration,[4–6] and those in which the temperature is maintained externally by incubation of small laboratory silos.[7,8] The results are variable and partly contradictory and this may arise from the fact that the causes of increases in temperature are complex and depend upon a number of factors. Because of these variables it is possible that two silages made at similar temperatures may give widely different results in terms of losses and chemical changes.

Experiments have been in progress in Edinburgh for a number of years with experimental metal silos (153 cm diameter × 182 cm high), each having a maximum capacity of 3·47 m.[3] Previous attempts to achieve temperatures above about 30° have been unsuccessful because of loss of heat through the silo walls. In the experiments reported here it was possible to control the silage temperatures by electrothermal insulation of the silo walls.

Experimental

The design of the silo unit consisting of four silos, and the bacteriological and chemical analyses have been described in earlier publications.[6,9] Preliminary tests showed that the four silos, filled under identical conditions with *Lolium multiflorum* (Italian ryegrass), yielded similar results.[6] For the purpose of this experiment two of the silos (A and C) were wrapped with 'Pyrotenax' heating cables (loading 132 W/m^2) and lagged with ½ in. fibreglass sheeting. The temperature of the metal wall of each silo was controlled by means of a thermostat, linked to the heating cable, and preset at 45–49°. Heating of the silo wall was maintained until the 8th day after filling, when the current was switched off. The other two silos (B and D) were not insulated.

The grass used was predominantly a mixture of two species, timothy (*Phleum pratense*) and meadow fescue (*Festuca pratensis*). Silos A and B were filled with 835 kg (127 kg of dry matter) of bruised, fresh grass; and silos C and D were filled with 835 kg (223 kg of dry matter) of bruised, wilted grass.

The herbage ensiled in silos A and B was cut on 10.6.64 with a flail-type forage harvester (Lundell 60, rotor speed 1200 rpm) and ensiled the same day. Sufficient grass was cut at the same time with a mower and allowed to wilt in the field for 27 h before lifting with the forage harvester and ensiling in silos C and D. During filling eight thermocouples were buried at different levels throughout the herbage and temperatures were subsequently recorded daily.

The herbage in the insulated silos A and C was only moderately compacted during filling and consolidation weights (273 kg), equivalent to a surface pressure of 14·8 g/cm^2, were applied on the second day. The grass in silos B and D was, however, well compacted and consolidation weights (273 kg) were applied immediately after filling.

Effluents were not produced from the silos containing wilted herbage; those from silos A and B were collected daily and stored at −18° until required for analysis.

Vertical core samples were taken daily, for pH, water-soluble carbohydrate content and bacteriological examination, during the first week after filling and at weekly intervals thereafter.

Digestibility trials and intake studies were carried out with Cheviot sheep on the original herbage and silages as outlined in previous publications.[6,9]

Results

Volume and temperature changes

The volumes occupied by the ensiled material during the first 8 days are shown in Table I. As expected, the volumes of the fresh herbages fell more rapidly than those of the wilted materials.

The silage temperatures in silos B and D remained relatively low, the maximum mean temperatures recorded being 20° and 24° on the 2nd and 4th days respectively. In the insu-

TABLE I
Volumes (m^3) occupied by silages

Day	Silage A	B	C	D
1	3·29	1·83	3·47	2·73
2	3·29	1·65	3·47	2·73
3	1·65	1·52	2·62	2·47
4	1·56	1·38	2·29	2·47
5	1·38	1·38	2·29	2·38
6	1·38	1·38	2·29	2·34
56	1·25	1·16	2·20	1·97

lated silos, the maximum mean temperatures were recorded on the 3rd day for silo A (42°), and on the 7th day for silo C, (37°). The detailed results are shown in Fig. 1.

Composition

The composition of the grass and silages is given in Table II. The original dry matter of the fresh grass was 15·2% and this was increased by wilting to 26·7%. Protein was the main constituent apparently affected by the wilting and this decreased slightly. Silage A was of poorest quality when assessed in terms of pH (4·57) and organic acids. The lactic acid content was extremely low (0·45%) and n- and iso-butyric acids, as well as a number of higher, volatile fatty acids, were present. Formic acid was also detected in this silage. The low-temperature silages were well preserved (pH 3·72–3·88) and contained appreciable amounts of lactic acid (7·93–7·41%). Acetic acid was the only other fermentation acid present in any quantity. Silage C was also well preserved (pH 4·06) but contained less lactic acid (4·77%) than the low-temperature silages, and traces of higher volatile acids were present.

The pH values of the cored silage samples are given in Fig. 2 and the most interesting feature of these results is the changes which occurred in silage A. The pH of the ensiled material fell from 5·90 to 4·28 by the 4th day and then steadily increased to 4·79 by the 20th day. The most rapid drop in pH was that which occurred in silo B where the value fell to 3·98 by the 6th day. The water-soluble carbohydrate (WSC) contents of the cored samples are shown in Fig. 3.

TABLE II
Composition of grass and silages
(% of true dry matter)

	Grass		Silages			
	Fresh	Wilted	A	B	C	D
Dry matter	15·21	26·71	16·20	16·89	25·49	24·79
Organic matter	91·8	92·5	92·3	92·4	92·1	91·9
Crude protein	12·8	11·3	13·3	13·0	11·7	12·0
Ether extract	2·7	2·5	3·3	3·6	3·6	3·8
Crude fibre	29·4	30·9	34·2	33·3	31·7	32·6
Total N	2·04	1·81	2·12	2·09	1·87	1·92
Protein N	1·68	1·42	0·95	0·88	0·69	0·63
Non-protein N	0·36	0·39	1·17	1·21	1·18	1·29
Volatile N	—	—	0·26	0·14	0·14	0·13
Water-soluble carbohydrates	8·31	8·07	0·75	1·17	4·18	1·63
Cellulose	32·7	33·5	36·8	35·2	34·2	34·8
Lignin	5·71	6·05	6·18	5·69	6·55	6·18
Formic acid	—	—	0·20	—	—	—
Acetic acid	—	—	4·53	2·67	1·11	1·42
Propionic acid	—	—	0·30	0·09	trace	trace
n-Butyric acid	—	—	0·51	—	trace	—
Isobutyric acid	—	—	0·07	—	—	—
n-Valeric acid	—	—	0·03	—	trace	—
Isovaleric acid	—	—	0·03	—	trace	—
n-Hexanoic acid	—	—	0·09	—	—	—
Lactic acid	—	—	0·45	7·93	4·77	7·41
Succinic acid	—	—	0·44	0·12	0·16	0·16
pH	5·90	6·00	4·57	3·72	4·06	3·88

Losses

The total fresh weights of silage removed from the silos were A, 624; B, 640; C, 813; and D, 822 kg and the losses calculated from these weights and the composition figures are given in Table III. Surface waste was weighed separately and this amounted to A, 19·2; B, 11·2; C, 19·4; and D, 8·1%, of the silages removed.

Gaseous losses, as a percentage of the fresh weight ensiled, shown in Fig. 4, were A, 16·5; B, 10·9; C, 9·9; and D, 10·3%. The losses of nitrogenous compounds from silos A and B were considerably greater than those occurring from the two wilted silages, most of this loss appearing in the effluent.

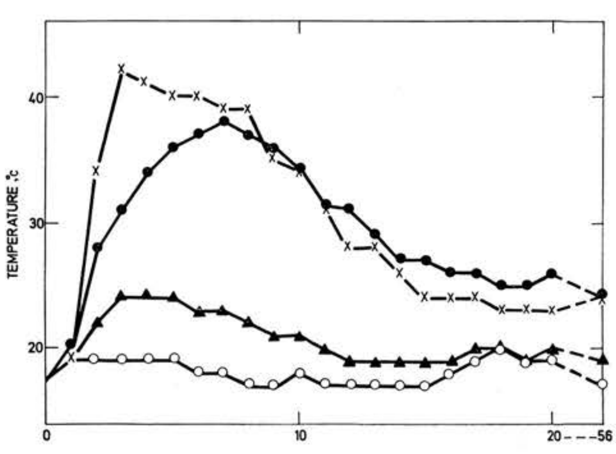

FIG. 1. *Temperature variation in silage*
(mean values for eight thermocouples)
× silo A ○ silo B ● silo C ▲ silo D

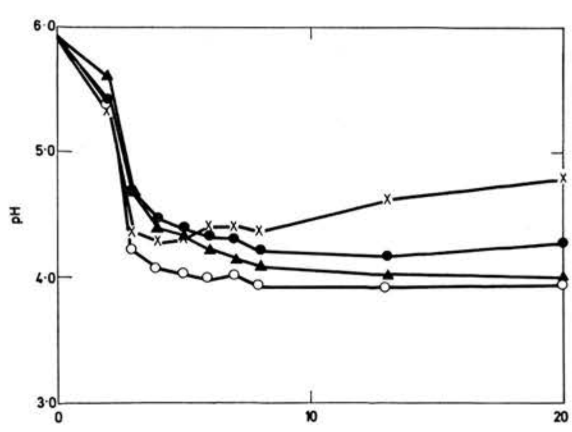

FIG. 2. *pH values of cored samples*
× silo A ○ silo B ● silo C ▲ silo D

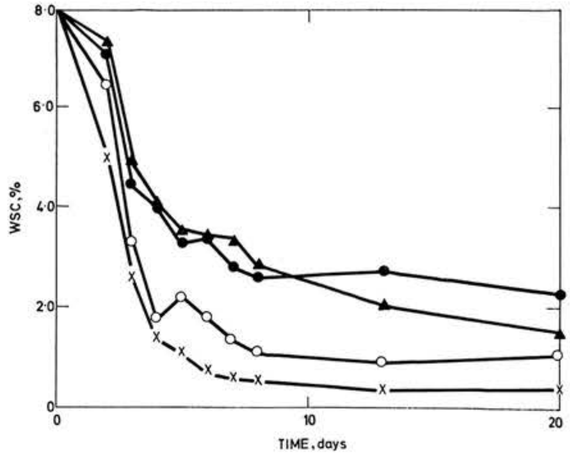

FIG. 3. *Water-soluble carbohydrate (WSC) contents of cored samples of dry matter (%)*
× silo A ○ silo B ● silo C ▲ silo D

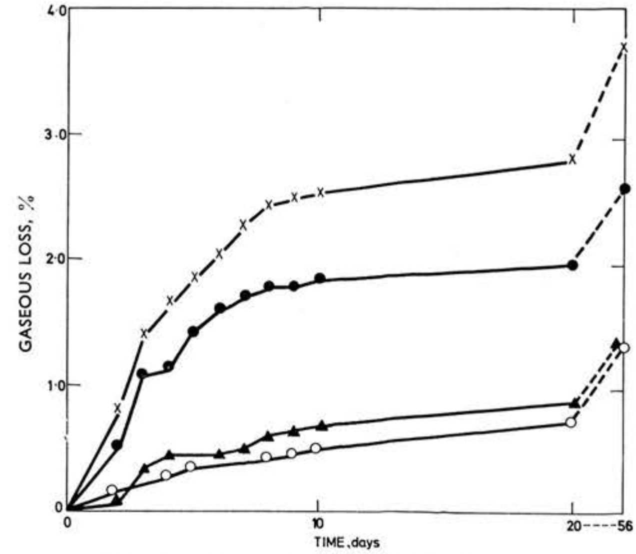

FIG. 4. *Gaseous losses as % of fresh grass*
× silo A ○ silo B ● silo C ▲ silo D

TABLE III
Percentage losses during ensilage

	A		B		C		D	
	Total	Effluent	Total	Effluent	Total	Effluent	Total	Effluent
Dry matter	21·4	4·9	16·2	5·2	9·9	—	10·3	—
Total N	18·3	10·1	14·3	9·4	6·8	—	4·6	—
Protein N	55·4	—	55·9	—	56·2	—	60·3	—
Water-soluble carbohydrates	92·9	2·1	88·2	3·6	54·7	—	82·4	—

Bacteriological studies

Counts of lactic acid bacteria and clostridia were made on cored samples taken at 3, 5, 8, 20 and 59 days after ensiling. All silages contained high numbers of lactic acid bacteria on each examination, varying from 10 to 150 million/g wet weight, increasing during the first 20 days with about a tenfold decrease at opening. Clostridia, on the other hand, were only encountered in the two high-temperature silages A and C. Only in the wet silage A, however, did they appear to occur in appreciable numbers, being detected in concentrations up to 100,000/g during the first few days. In the wilted silage C, they were present at the level of 1000/g at 20 days, not being detected previously.

Nutritive value

The digestibility coefficients and digestible nutrients are shown in Table IV. The mean daily dry matter intakes (g/kgW$^{0·73}$) were: fresh grass 78; wilted grass 76; silage A 66; silage B 65; silage C 55; silage D 52.

Discussion

The main source of heat produced during ensilage arises from aerobic respiration with only a relatively minor contribution from anaerobic fermentation. The increase in temperature which occurs in the silage mass depends upon the extent and rate of respiration, the degree of insulation of the silo and the specific heat of the ensiled mass. The herbage in the insulated silos A and C was only moderately compacted during filling and consolidation weights (273 kg), equivalent to a surface pressure of 14·8 g/cm^2, were applied on the second day. This treatment was designed to allow air to penetrate the herbage and resulted in a marked temperature increase. The extent of respiration is controlled mainly by the oxygen supply and the available sugars, but the rate of respiration is affected by the temperature. As far as the plant is concerned, within the range 0–30°, the acceleration of respiration with temperature is roughly exponential.[10] Above 30°, the rate of increase per unit of temperature slowly decreases, but rise in temperature still accelerates respiration until the enzymes

TABLE IV
Percentage digestibility (D), percentage of digestible nutrients (DN) and energy values of true dry matter
(mean values)

	Grass				*Silages*							
	Fresh		Wilted		A		B		C		D	
	D	DN	D	DN	D	DN	D	DN	D	DN	D	DN
Organic matter	71·9	66·1	72·8	67·3	74·0	68·2	75·6	69·9	75·8	69·8	73·2	67·3
Crude protein	69·2	8·8	65·5	7·4	69·3	9·2	71·8	9·4	66·1	7·7	64·8	7·8
*M.E. (kcal/g)		2·41		2·46		2·52		2·59		2·59		2·51
Starch equivalent		58·3		56·9		59·6		61·8		60·5		57·7

* Metabolisable energy. Calculated from total digestible nutrients (TDN) on the assumption that 1 g of TDN has an M.E. value of 3·559 kcal.

are inactivated. In a farm silo it is reasonable to assume that by the nature of its size, considerable insulation of the central mass will occur. This will apply in particular to dry silages. In experimental silos of the type used in these studies it was found necessary to prevent loss of heat by electrothermal insulation. Although this method of preventing heat loss is not ideal, since some heat must inevitably be absorbed by the silage mass from the heated metal walls, previous experience had shown that this was the only satisfactory method of achieving temperatures within the range 35/45°.

The specific heat of dried grass, prepared from fresh herbage used in this experiment, was estimated from calorimetric studies, to be of the order of 0·45. From this it can be calculated that the specific heat of the fresh grass ensiled in silos A and B would be approximately 0·92 and that of the wilted material 0·85.

Oxygen, rather than sugar, will normally be the limiting factor in controlling the extent of respiration in a sealed silo. In the experiment reported here, silo B was rapidly consolidated and the surface of the herbage was covered with plastic sheeting. The initial volume of oxygen trapped in the mass of fresh grass (835 kg) occupying a volume of 3·02 m^3 was calculated to be 0·44 m^3. If all this oxygen was used for the complete oxidation of sugar (glucose), then the heat produced in an insulated silo would cause a temperature increase in the mass of only 2·8°, and this increase could be brought about by the oxidation of 0·07% glucose in the fresh herbage (equivalent to 0·46% glucose in the herbage dry matter).

The actual temperature increase in silo B was 1·5° (17·5°–19·0°) which is less than the value calculated above. Silo B, however, was not insulated and it is likely that some heat loss through the metal walls took place. The presence of some surface waste at the end of the ensiling period indicated that air had penetrated the upper layers of the mass and suggested that the sugar loss was slightly greater than that estimated above. The presence of a relatively large amount of lactic acid in the silage confirmed, however, that most of the sugars had in fact been fermented rather than oxidised.

In the foregoing calculations the heat of combustion of glucose (673 kcal/mole) has been used. According to Wohl & James,[11] the release of total energy in respiration leads to its escape from the plant as heat to the amount of almost 100% during the mature phase of growth and only falls short of 100% during the growth phase to the extent that energy is fixed by the reactions of synthesis. In the harvested plant, chemical reactions are mainly katabolic and it is therefore likely that the energy from hexose oxidation is entirely liberated as heat.

In silo A, a temperature rise of 24·5° was recorded by the third day and, assuming that the heat was produced entirely from aerobic respiration of sugars, a minimum of 4·0% glucose (in herbage dry matter), or about half of the original WSC content, would be required for this temperature increase.

The results in Fig. 3 show that by the third day 5·5% WSC in the herbage dry matter had in fact disappeared. Available sugar was clearly a limiting factor for subsequent lactic acid production and, since a sufficiently low pH value had not been obtained to ensure preservation of the material, a clostridial fermentation had occurred with consequent destruction of lactic acid and a subsequent breakdown of amino-acids resulting in the formation of n- and iso-butyric and valeric acids. Amino-acid breakdown is also reflected in the relatively high volatile-N figure given for silage A in Table II.

The effect of temperature on respiration rate has already been mentioned; of equal interest is the direct and indirect effects of temperature on bacterial activity. In the first instance, the rate of bacterial metabolism is generally increased with increase in temperature up to about 40°. As the temperature reaches 40°, however, a selective effect will take place on the bacterial population, many lactic acid-forming bacteria being unable to multiply at this or at slightly higher temperatures. A successful fermentation will then depend upon the presence of one or more of the very few types of lactic acid bacteria able to grow actively within the range 40–50°. The indirect effect on bacterial activity relates to the amount of residual sugars which are available after respiration losses and this has already been described.

The differences shown between the two wet silages were not reflected to the same degree with the wilted materials, although the low-temperature silage was of slightly lower pH value and contained more lactic acid than the high-temperature silage. Traces of higher volatile fatty acids in the high-temperature-wilted material indicated that some amino-acid breakdown had occurred and this conclusion was in agreement with the bacteriological results in which low numbers of clostridia were detected. The clostridial activity, however, had clearly been slight and had had little significant effect on the preservation.

The water-soluble carbohydrate contents of the two wilted silages were higher than those of the wetter materials. This finding for wilted materials has been confirmed in a subsequent experiment. The much higher value for silage C is, however, difficult to explain and the nature of the carbohydrate fraction in wilted silages is being investigated further.

In practice, bad silage is frequently associated with low temperatures and high moisture content. Stress has, perhaps, in the past been laid more upon temperature as a factor influencing fermentation, rather than moisture, although the two are clearly inter-related in that temperature is usually an indication of the wetness of the crop. Attempts to increase the temperature of a wet crop by delayed filling and aeration result in a rapid loss of soluble sugars by respiration and in a crop of low WSC content there is likely to be a deficiency of sugars for subsequent fermentation.

The advantages of wilting crops prior to ensiling are well known. The major benefit is the effect on clostridial growth. The clostridia are relatively intolerant of dry conditions, when compared with lactic acid bacteria,[12] and consequently the preservation of a wilted crop is not so dependent upon the production of a low pH value. Under practical conditions it is well known that it is more difficult to prevent overheating in wilted than in fresh material. This is, however, a reflection of the difficulty in consolidating the crop and preventing re-entry of air. In our experiment, in the insulated silos the wet material heated more rapidly than the wilted material, which suggests that enzyme activity was greater in the wet material as would be expected. Most of the heat would have resulted from plant enzyme activity, since in the early stages of ensilage the heat produced by microbial respiration is considered to be small.[8] Oxygen was a limiting factor by the second day because of heavy consolidation and because of this the temperature of the wilted herbage was more easily controlled.

Feeding trial

There is no evidence from the feeding trials that the silages differed markedly in nutritive value, apart from a small increase in digestible crude protein content in the wet silages. This is presumably a reflection of the higher crude protein content of the wet silages compared with the wilted materials. There is also no evidence from the intake figures that the sheep showed any preference for either of the wet silages, but the lower intakes by animals on the wilted silages are contrary to the findings from other published work.[13] The dry matter contents of our wilted silages, however, were relatively low and too much importance cannot be attached to these results.

Conclusion

It has been shown that the conditions of ensilage can affect markedly the subsequent fermentation, and the importance of conserving the sugars for lactic acid fermentation has been demonstrated. It would seem that a low temperature and the use of wilted herbage leads to the production of a silage least likely to be spoilt by clostridial development.

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16.

CHANGES IN ORGANIC ACID COMPOSITION DURING INCUBATION OF ASEPTICALLY-GROWN GRASS

By M. J. PLAYNE*, ANNA C. STIRLING and P. McDONALD

The organic acid composition of aseptically-grown timothy grass (*Phleum pratense*) was examined. Changes in organic acids of the grass during incubation in small test tubes with and without the addition of lactic acid bacteria were studied. Malic acid, a major acid of the grass, was broken down during incubation mainly by the activity of bacterial enzymes. The amount of succinic acid was increased by the action of both plant and bacterial enzymes. Citric acid was not detected in the aseptically-grown timothy grass but two unidentified acids formed a major part of the total organic acid fraction.

Introduction

The breakdown of some plant organic acids (malic, citric) has been shown to occur during ensilage.[1,2,3] Although many bacteria found in silage are able to dissimilate these acids[4,5] the respective rôles of plant and bacterial enzymes have not been clearly demonstrated. Increases in succinic acid in addition to the production of lactic and acetic acids during ensilage have been reported.[1-3] Succinate appears to accumulate in higher plants under conditions of anaerobiosis and high carbon dioxide concentration,[6] while its production from malate by the action of a lactobacillus at a pH above 5 has been shown.[7] In this study, changes in the organic acid composition after incubation of aseptically-grown timothy grass (*Phleum pratense*) in the presence or absence of certain lactic acid bacteria were recorded.

Experimental

The procedure for the production and harvesting of bacteria-free grass was that developed by Stirling,[8] derived from the method of Mabbitt.[9,10] Sterilised seeds of timothy grass were grown over sterile nutrient solution in glass tubes each of which was tested for sterility before harvesting.

Five small tubes each holding approximately 10 g were filled with the grass, weighed, stoppered with sterile bungs carrying mercury valves and held in a water bath at 30°. Three of the tubes were inoculated with silage bacteria, *Streptococcus faecalis*, *Streptococcus faecium* or *Lactobacillus plantarum*.

After 10 days the tubes were opened aseptically, weights were recorded and the contents of each tube macerated with 40 ml sterile water to provide material for bacteriological and chemical analysis.

Analytical methods

The pH, buffering capacity and organic acids were determined by the methods described in a previous publication.[2] The dry matter of the grass was determined by drying 10 g at 75° for 16 h. The results for incubated material are expressed on the basis of tubed plant dry matter. Water soluble carbohydrates (WSC) were determined by the method of McDonald and Henderson.[11]

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Results

The dry matter of the aseptically-grown grass was 11% and the water soluble carbohydrate content was 4% of the dry matter. The figures given in Table I show that the buffering capacity increased markedly on incubation despite a rise in pH in all the incubated material.

The organic acid composition of the fresh and incubated plant material is given in Table II. The outstanding feature was the presence of large quantities of two unidentified acids which were eluted together from the column and which formed about 70% of the total organic acids found. Both were eluted in one broad peak emerging in approximately the position of citric acid, but when chromatographed on paper, two acid spots appeared neither of which was citric acid. The position of the acids on the column and their movement on paper using two solvent systems did not conform with those of any of the common plant acids.

Malic was quantitatively the most important of the other acids although the amount decreased slightly with incubation, while succinic and acetic acids both increased. Butyric, lactic, propionic and formic acids were not found.

In the inoculated material (Table II) malate was broken down completely by *S. faecalis* and partly by *S. faecium* and *L. plantarum*. The unidentified acids comprised 45–52% of the total acids. Formic acid was detected only in the material inoculated with *S. faecalis*. The malonic/lactic peak in all three samples contained predominantly lactic acid. The succinic acid content was high in all inoculated material and acetic acid was increased with *S. faecalis* and *L. plantarum*. The content of acetic acid exceeded that of lactic acid with *L. plantarum*.

Table I

pH and buffering capacity values of aseptically-grown grass

Material	pH	Buffering capacity (pH 4–6) m-equiv./100 g dry matter
Fresh grass	6·18	28·8
Incubated grass 1	7·17	40·6
Incubated grass 2	7·28	46·7
Inoculated grass with:—		
S. faecalis	6·64	68·9
S. faecium	7·16	93·2
L. plantarum	6·85	115·5

TABLE II

Organic acids in inoculated grass after incubation
(m-equiv./100 g dry matter)

Acid	Fresh Grass	Grass incubated for 10 days 1.	2.	Grass inoculated with: *S. faecalis*	*S. faecium*	*L. plantarum*
Valeric	—	—	—	0·26	Nil	0·19
Acetic	0·43	1·62	1·26	5·41	1·66	15·26
Fumaric	2·51	4·41	2·04	9·08	2·77	2·09
Formic	—	—	—			
Succinic	0·89	3·12	3·47	4·75	4·56	8·46
Malonic	1·79	1·91	1·99	35·71	23·97	12·97
Lactic	—	—	—			
Glycollic	0·85	Nil	Nil	0·88	0·29	0·50
Malic	18·16	13·55	11·57	Nil	8·16	2·58
Unidentified (2 acids)	58·34	40·59	52·22	47·14	44·57	34·74
Total	82·96	65·19	72·54	103·22	85·97	76·78
Total excl. unidentified acids	24·62	24·60	20·33	56·08	41·40	41·85

Bacterial counts were made on all incubated material. These were with *S. faecalis*, 730; *S. faecium*, 460; and *L. plantarum*, 230 (million per g dry matter). Bacteria were not detected in the other materials.

Discussion

Malate was apparently degraded to a slight extent when bacteria-free grass was incubated, but the decrease in quantity was small compared to the decrease observed in the inoculated grass, or to that which has been observed in silage.[1,2] Bacterial enzymes appear, therefore, to be mainly responsible. All three strains of bacteria used for inoculation were known to be capable of dissimilating malate.

The occurrence of formate only in the grass inoculated with *S. faecalis* indicated phosphoroclastic activity and the use of malate as an energy source by this organism. This finding is in agreement with the results of previous studies.[12,13]

The increase of succinate appears to be largely caused by plant enzymes except in the case of *L. plantarum* incubate. This increase probably occurred at the expense of malate during the early stages of incubation when oxygen was present.

Citrate is of almost universal occurrence in grasses and the absence of citrate from the aseptically-grown timothy grass is thus surprising. The remaining identified organic acids do not, however, differ markedly in content from those reported in a previous publication for Italian ryegrass.[2] A recent examination of a sample of timothy grass grown under field conditions[14] showed the presence of malic, citric, fumaric, glycollic and succinic acids with malic being the dominant acid. The unidentified acids present in the aseptically-grown herbage were not found in the field-grown timothy, which suggests that these acids may have resulted from the abnormal conditions of growth of the experimental grass.

The WSC content of the aseptically-grown herbage was low (4% of the dry matter) although this is not necessarily abnormal since values as low as this have been reported for timothy grown under field conditions in S.E.Scotland harvested in September.[15] The low WSC content was clearly insufficient to allow lactic acid bacteria to produce a low pH value on incubation.

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The Edinburgh School of Agriculture

The Principles of Ensilage

By P. McDONALD, S. J. WATSON and R. WHITTENBURY

In the preservation of a green crop as silage, the main object is to store the material with a minimum loss of nutrients. In order to do this the first essential is to achieve and maintain anaerobic conditions, otherwise considerable loss will occur from the oxidative activities of plant enzymes and aerobic micro-organisms. Secondly the aim is to discourage a clostridial fermentation since this results in the production of carbon dioxide, ammonia and nitrogenous compounds such as amines which may be toxic.

In practice anaerobic conditions can be achieved by various methods. The most efficient way would be to store material in a hermetically sealed container, and under these conditions the oxygen, trapped by the herbage, will be consumed rapidly with insignificant losses of sugars. In the open-type silo, the efficiency with which anaerobiosis can be obtained will depend upon the degree of consolidation and final sealing. The main aim of consolidation and sealing in this type of silo is to prevent re-entry and circulation of air during storage. Where air is in contact with cut herbage for any period of time, then as a result of aerobic microbial activity, the material decays to a useless, inedible, and sometimes toxic product.

With regard to the second objective there are two main ways in which a clostridial type fermentation can be reduced to a minimum. The most direct way of achieving this is to reduce the moisture content of the crop. In ensiled crops containing 30 per cent, or more, dry matter very little clostridial activity occurs and the crops preserve satisfactorily. Clostridia are particularly sensitive to water availability and they require very wet conditions for active development. The commonest way of controlling clostridial growth is to promote a lactic acid fermentation. The lactic acid and acetic acid, which are formed, increase the hydrogen ion concentration to a level at which clostridial development is inhibited. It should be emphasised, however, that the inhibitory effect is caused by both hydrogen ion concentration and undissociated acids themselves. Consequently, it is not possible to state an exact 'critical pH level' for silage; this depends not only on the pH level but also on the moisture content and the temperature. The wetter the material the lower will be the critical pH value. Under dry conditions a lactic acid type fermentation will normally occur; this, however, is not necessarily essential for preservation, but is an inevitable consequence of the existing conditions.

An alternative and logical method of achieving a stable pH value is by the direct addition of mineral acids as in the A.I.V. process named after its originator A. I. VIRTANEN. This method, which relies solely upon the hydrogen ion effect for preservation, is not popular in some countries because of the practical difficulties in handling the acid.

The above presents a brief introduction to the ensilage process. While the authors are fully aware of the valuable contributions made by many other workers in this field, the aim in this paper is not to review the literature, since this has been done elsewhere (1, 30) but to present the conclusions of a team of workers in Edinburgh who have during the past 15 years made some contribution to an understanding of the ensilage processes. Attention in this work has been concentrated more upon the natural process than on methods involving the use of additives. This is essential to any fundamental approach since the function of an additive is to stimulate or to inhibit a natural process, which must be properly understood before it can be controlled. The extensive nature of this work has been made possible only by a very generous grant to the University of Edinburgh by the Agricultural Research Council.

Equipment and techniques

In order to study the biochemical and microbial changes and to measure the losses during ensilage it was first necessary to devise new techniques and equipment. Both field and laboratory scale experiments were conducted. For the former a silo unit was constructed which allowed the continuous measurement of effluent and gaseous losses and permitted of daily recordings of pH and temperature changes. The unit consisted of four metal silos, each having a maximum capacity of 1000 g fresh herbage, and each being suspended from a weighing device (2, 3).

The carbohydrate components of herbage assume special importance in ensilage and paper chromatographic techniques have been adapted to enable the quantitative measurement of individual carbohydrates in grasses, legumes and silages to be made (4–10). Similarly, techniques using paper chromatography, ionophoresis and ion exchange column chromatography have been developed in order to measure the nitrogenous products of protein breakdown (11–18). During the course of this work, the organic acids assumed special importance (19), particularly in connection with the buffering properties of herbage (20, 21), and a method for the determination of these acids using a continuous gradient elution system has recently been devised (22). The accurate determination of dry matter is of great importance in studying losses during ensilage and a simple method based on toluene distillation has been developed (23, 24).

Techniques were also developed for studying bacteriological changes in test tube silos and for isolating and growing different types of bacteria involved in silage fermentation (25–37). In order to study the specific effect of the bacteria and plant enzymes methods were developed for growing grass aseptically (38, 39).

Nature of the Crop

Chemical composition

In order to have a clear understanding of the fermentation process it is necessary first to characterise herbage on the basis of its chemical composition. The level of fermentable carbohydrates available to the micro-organisms is of basic importance, especially in wet crops, to ensure that adequate lactic acid bacterial development occurs. The main fermentable carbohydrates include glucose, fructose, sucrose, melibiose, raffinose and in grasses, but not in legumes, fructosans (3). These carbohydrates, which are all extracted by cold water, are collectively designated 'water soluble carbohydrates' (WSC) and can be determined by a routine iodometric method (40). The WSC content of herbage varies with species (41–43), stage of growth (10), sunshine (10), and fertilizer application. Work is at present in progress to investigate further some of these factors. Generally, under conditions of growth in S.E. Scotland, legumes are frequently low in WSC content while of the common species of grasses, Italian ryegrass *(Lolium multiflorum)* is normally the richest and cocksfoot *(Dactylis glomerata)* frequently the lowest. WSC values for Italian ryegrass have exceeded 30 per cent (dry matter basis) while values recorded for cocksfoot have been less than 4 per cent in some instances. The structural carbohydrates play little part in the ensilage process although hemicelluloses may make a minor contribution (9) through the action of plant hemicellulases (44). Of the total nitrogen present in fresh herbage, generally 85–90 per cent is protein, the remainder being largely free amino acids. On harvesting, proteolysis occurs rapidly, due to plant enzymes, and is only inhibited by acidification or rapid removal of moisture.

Most of the tricarboxylic acid cycle acids have been detected in red clover and ryegrass (21, 22). These acids, particularly citric and malic, play an important role as buffers in the plant (22) and not proteins as was previously thought. The relatively high buffering capacity of legumes (20) is one reason why these crops are difficult to ensile satisfactorily.

The importance of moisture has already been discussed and it is clearly important to avoid ensiling wet crops. Unfortunately in many areas, wilting is often difficult although it has been shown in S.E. Scotland that even in poor weather (cool, overcast, with occasional showers) moisture loss still occurs (45–47).

Physical state of the crop

The introduction of the forage harvester has undoubtedly simplified silage making. Herbage that has been bruised and lacerated consolidates more easily and the sap containing sugars and other nutrients essential for the growth of lactic acid bacteria is immediately liberated thereby enabling fermentation to proceed rapidly (43, 48).

Microbial flora

The changes brought about in the ensiled herbage are largely caused by bacteria, consequently the types and numbers of bacteria on the plant material assume importance (30). The great majority are strict aerobes and contribute little or nothing to silage preservation. Of the organisms able to grow anaerobically, coliforms are the most numerous but are not important agents in the preservation processes. Neither *Bacillus* nor *Clostridium* occur in large numbers on fresh material. *Streptococcus, Leuconostoc, Pediococcus* and *Lactobacillus*, the organisms primarily responsible for the production of lactic acid, appear to be relatively scarce (32). On the occasions that high numbers have been found, it has been because the material has not been freshly cut, multiplication occurring during the wilting and harvesting of the crop. As well as bacteria, yeasts sometimes develop in ensilage, but do not appear either to harm or contribute to preservation.

The final bacteriological quality of the crop is influenced by the implements used to ensile it (32). On farms with a history of ensilage, additional lactic acid bacteria, amongst other micro-organisms, may be added to the herbage.

Changes during ensilage

Once the material has been ensiled a regular sequence of events takes place. The herbage quickly consumes the entrapped oxygen and the aerobic micro-organisms die rapidly. Bacteria capable of anaerobic growth now proceed to multiply immediately if they are present (species of *Streptococcus, Leuconostoc, Pediococcus, Lactobacillus, Clostridium* and *Bacillus, Bacterium herbicola* and the coliform group). The active multiplication period lasts for 2 to 3 days only in the temperature range 20 to 40° C, and is then followed by a phase of declining count. The rate of growth and final density of the bacterial population (48) varies with the quality of the herbage, whether or not it is bruised and lacerated, and the temperature (28).

Gram-negative rods (coliforms) are normally the first group to dominate the bacterial flora, forming mainly acetic acid and carbon dioxide, and these are followed by streptococci and leuconostocs which eventually succumb to the high acidity which normally develops; the flora is finally dominated by the acid-tolerant lactobacilli and pediococci. Under ideal conditions the pH value will decrease to about 4.0 and the subsequent microbial development is negligible. The flora of silages late in the storage period may or may not be dominated by some particular lactic acid bacterium; however, it has proved useless to try and predict earlier events or to assess the quality of the silage by examining the microbial flora at the end of the ensiling period.

Should the pH of a wet silage not reach a critical level for preservation, events take a different course from that described. Clostridia will not be inhibited and certain species will continue to proliferate on the lactate and residual sugars, with the formation of butyric acid and other volatile fatty acids. As this involves decarboxylation of lactate, the hydrogen ion concentration is decreased: so creating a favourable environment for other clostridia which will now attack the amino acids. Decarboxylation of these acids results in the formation of the amines, histamine, tyramine, putrescine and cadaverine, while deamination produces straight and branched-chain fatty acids and free ammonia (17, 18). In high pH silages the ammonia may reach an extremely high figure accounting for 50 per cent or more of the non-protein nitrogen fraction.

In a well preserved silage, lactic acid forming bacteria are without doubt the most important organisms and their metabolism has been and is being investigated in great detail. It is commonly assumed that all their activities are beneficial, although it is realised that some lactic acid bacteria are more useful than others. Investigations reveal that the situation is complex. The metabolism of lactic acid bacteria on hexoses and related sugars is one of two types, homofermentative, where ideally a molecule of glucose is fermented to two molecules of lactic acid, and heterofermentative, where one molecule of glucose is converted to one molecule of lactic acid, one of ethanol and one of carbon dioxide. Different products, however, result from the fermentation of fructose by this latter group of organisms (50). Here, ethanol is replaced by acetate and much of the fructose may be reduced to mannitol. Other neutral products such as diacetyl and acetoin may be formed. Clearly the heterofermentative bacteria produce less lactic acid per molecule from fructose than glucose. Experiments are in progress to study some of these pathways in further detail.

The bacterial action on the plant organic acids mentioned earlier is not well understood, but present studies suggest that malates and citrates are extensively broken down by lactic acid bacteria. The products formed are numerous, amongst them being lactate, acetate and formate. These are present as salts and contribute towards the buffering properties of the silage and act against a fall in pH. This explains why it is often difficult to relate total lactic acid content to pH value. Other products (36) from organic acids are either neutral, (ethanol, diacetyl, acetoin, 2, 3 – butanediol) or alkaline (bicarbonates and carbonates of major plant elements) which can neutralise some of the free acids formed from sugars.

Obviously not all the activities of the lactic acid bacteria are beneficial in ensilage. In low sugar/high organic acid material the action of the lactic acid bacteria and the coliforms may interfere with the acidic preservation of the material. In such cases moisture content can be of paramount importance and wilting may be the only natural way of preventing clostridial growth.

Action on amino acids by lactic acid bacteria appears to be restricted to serine and arginine. In this instance, deamination contributes to volatile losses.

Losses during ensilage

Losses of nutrients during ensilage occur as a result of four main factors, – effluent flow, aerobic plant enzyme activity, aerobic microbial activity and the action of anaerobic micro-organisms. Effluent loss is related to moisture content of the crop (3, 41) but it is also increased by the formation of metabolic water derived from respiration. In a wet crop free drainage is important, since the retention of the effluent in the bottom of a silo encourages clostridial development in spite of an apparently favourable pH value. Dry matter losses via the effluent can exceed 10 per cent (43). The obvious way of preventing this loss of valuable nutrients is by wilting the crop, preferably to at least 30 per cent dry matter before ensiling. Secondly, losses of valuable sugars from aerobic plant respiration can be reduced to a minimum by filling, adequate compaction and prevention of re-entry of air during storage.

Aerobic microbial activity takes place where the surface is exposed to air. Surface waste can frequently appear to be small although in its production as much as 50 per cent of the herbage dry matter may be lost as gaseous products (43). The action of aerobic micro-organisms can be completely eliminated by adequate sealing of all silage surfaces. Finally some losses of dry matter occur as a result of anaerobic fermentation. If a clostridial fermentation occurs then the losses will be high, resulting in the production of carbon dioxide and ammonia as mentioned earlier. The least gaseous loss will occur from a fermentation dominated by homofermentative lactic acid bacteria for the reasons given earlier. If the fermentation is due to heterofermentative lactic acid bacteria, then some carbon dioxide loss is inevitable, approximately one-sixth of the sugar (glucose) carbon being lost as carbon dioxide.

Temperature

Provided the ensiled crop is not excessively rich in moisture, the object should be to keep the temperature within the silos as low as possible. High temperatures result from the oxidation of sugars in plant respiration and frequently result in poor silages through a deficiency of sugars for subsequent fermentation. In the past bad silage has commonly been associated with low temperatures but it is possible that the latter are merely an indication of the degree of wetness of the crop. The only known advantage of allowing herbage to heat up during filling is a possible wilting effect at the silo, with a consequent increase in dry matter content. This, however, is a less efficient way of removing moisture than wilting in the field and it may reduce the available carbohydrates to below the initial level for adequate acid production.

Future objectives

In order to increase the efficiency of silage making on the farm a much more detailed knowledge of the variation in crop composition and microbial activity is necessary.

Great improvements can already be made by ensiling wilted crops under completely anaerobic conditions. Where wilting may be impracticable then alternative ways of restricting clostridial activity must be used. The development of dry sugar additives may be beneficial and the effects of inoculation with homofermentative lactic acid bacteria are being studied. Encouragement of a strictly homofermentative sugar fermentation is clearly desirable in the light of the previous discussion. Preliminary results suggest that the addition of both sugar and inoculum leads to a rapid pH fall.

Great stress in this paper has been laid on the organic acid content of herbage and this is of special importance in the ensilage of legume crops. For the reasons given earlier the wilting of legume crops prior to ensiling would appear to be of prime importance.

Many of the principles involved in the ensilage of green crops can be applied to the conservation of moist grain. Investigations are being conducted into the increasing practice of storing moist barley and oats for feeding purposes. Results indicate that strict anaerobiosis is sufficient in itself to achieve preservation. The relatively high dry matter (60–80 %) content of the grain prevents an active growth of clostridia, and a lactic acid type fermentation, whether it occurs or not, is not necessary.

To conclude, the two principles stated at the beginning of this paper are now accepted by the farmer. Where they are properly applied and proper care is taken in making silage the product has improved markedly in quality and feeding value of recent years. This general improvement has been greatly assisted by modern machinery, more particularly the flail harvester and by the trend towards some degree of wilting of the herbage before ensilage.

The more the ensilage process can be made to depend upon controlled natural changes the more acceptable it will become, and this depends entirely upon a proper knowledge of the biochemistry and microbiology of the process. There is still far too much work published where no proper deductions can be drawn since the basic information on the composition of the crop, its micro-flora, and the conditions in the silo is entirely lacking.

Zusammenfassung

Dieses Übersichtsreferat bringt Ergebnisse einer Arbeitsgruppe aus Edinburgh, die in den letzten 15 Jahren den Silierungsprozeß untersucht hat. Die Grundlagen der Silierung werden besprochen, und Angaben werden gemacht über Ausrüstung, chemische Methoden und bakteriologische Verfahren. Beschrieben werden Einflüsse solcher Faktoren, wie Feuchtigkeit und Temperatur einerseits, chemischer Zusammensetzung, physikalischer Beschaffenheit und Mikroflora des Ausgangsmaterials andererseits, auf den Silierungsprozeß. Die Entwicklung von Clostridien zu verhindern durch schnelle Erzeugung und Aufrechterhaltung anaerober Bedingungen und durch Erhöhung des Trockensubstanzgehaltes im Ausgangsmaterial auf bis zu 30 % wird als besonders wichtig angesehen.

Summary

This review article presents the conclusions of a team of Edinburgh workers who have been studying the ensilage process over the past fifteen years.

The basic principles of ensilage are discussed and references to the equipment, chemical methods and bacteriological techniques are given.

The effect of such factors as moisture, temperature, as well as the chemical composition, physical state and microbial flora of the original crop on the ensilage process are described.

Stress is laid upon the prevention of clostridial development by the rapid production and maintenance of anaerobic conditions and also by increasing the dry matter content of the original crop to 30 per cent or more.

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Some Biochemical and Microbiological Aspects of Ensilage

by R. Whittenbury, P. McDonald and D. G. Bryan-Jones

Summary

The products of the fermentation of soluble carbohydrates, organic acids and amino acids present in grasses have been examined, and have been shown to vary depending upon the type of bacteria responsible. The significance of the presence in silage of various volatile fatty acids, amines and neutral fermentation products such as mannitol and acetoin is discussed.

Introduction

The object of storing green crops as silage is to preserve the material with a minimum loss of nutrients. In this process, the first essential step is to achieve and maintain anaerobic conditions, thereby inhibiting the wasteful activities of aerobic microorganisms and oxidative enzymes of the plant material. The second step is to aim at the inhibition of protein destruction by clostridia under anaerobic conditions. Clostridial activity can be inhibited in silage by two methods. The most direct is to reduce the moisture content of the crop. If the dry matter is increased to about 30% by wilting in the field, subsequent clostridial activity in silage is slight as these bacteria are very sensitive to osmotic pressure and require very wet conditions for active growth. The second method is to allow an acidic fermentation to take place. Fortunately this occurs naturally; lactic acid forming bacteria produce a mixture of acetic and lactic acids, lactic acid predominating, from the water soluble carbohydrates. The combined effects of increased hydrogen ion concentration and the toxic nature of these acids at a low pH, result in the inhibition of clostridial development. It is not possible to state an exact pH value at which clostridia are inhibited since this depends upon several factors, particularly moisture content. Clostridia will tolerate high concentrations of organic acids and hydrogen ions under very wet conditions. Consequently the wetter the material, the lower will be the critical pH value for preservation.

An alternative and perhaps more certain method of preserving green crops under anaerobic conditions, is to add acids or other inhibitors directly to the plant material. Such is the basis of the A.I.V. process originated by Virtanen. In this country this method is unpopular because of the practical difficulties of handling the acid.

The purpose of this paper is to summarise the main fermentation changes which occur during the ensilage of herbage by the natural process. The products of the fermentation of soluble carbohydrates and organic acids referred to in this paper have been detected in silages using analytical techniques described in previous publications.[1, 2, 3, 4]

Soluble Carbohydrates

Glucose, fructose, sucrose and fructosans are the main water soluble carbohydrates (WSC) in grass, fructosans being the main reserve polysaccharides of the grasses. Oligosaccharides other than sucrose have been detected but it is doubtful if these are present in any significant quantity. Sucrose and fructosans are rapidly hydrolysed in ensilage to the monomers glucose and fructose. For microbiological pruposes we can consider that fructose and glucose are the major carbohydrate sources. The soluble carbohydrate content of green crops is extremely variable, depending upon species[5], stage of growth[6], weather[7], and fertiliser application[8]. In addition to variations in total water soluble carbohydrate content, the fructose/glucose ratio in grasses is also affected. Table 1 shows the variation in soluble carbohydrates of five samples of Italian ryegrass (Lolium multiflorum) used in silage studies and harvested at a young leafy stage of growth over a number of seasons. The fructose/glucose ratio in these samples ranges from 1.7 to 3.9. In a similar study using perennial ryegrass (L. perenne) Mackenzie & Wylam[7] found the fructose/glucose ratio varied from 1.1 to 3.7. It would appear that in the ryegrasses fructose is the dominant sugar. Attempts have been made to calculate sugar requirements for the satisfactory conservation of grass and legume crops. One recent suggestion is that a minimum of 6-7% (dry weight) fermentable hexose is essential to lower the pH value to 4.0[9]. A calculation of this type is based on an assumed efficiency of hexose fermentation by bacteria, the dissociation constant of the resulting acid, and an assumed buffering capacity. In our experience, such assumptions are liable to error as they do not take into account the variety of microbiological activity possible which is discussed below.

It cannot be assumed that all the WSC available in the crop will be fermented. In the process of ensilage substantial amounts may be lost through aerobic microbial oxidation and plant respiration. These losses clearly contribute to the frequently reported high "fermentation" losses around 30%. It is worth emphasizing here that "fermentation" losses of this order are impossible under silage conditions. True anaerobic losses will amount to no more than 6-8%[10]. These excessive losses should be referred to as oxidative losses as this would help to direct attention to the importance of keeping silos air-tight.

Soluble carbohydrates surviving aerobic metabolism are fermentatable by a variety of microorganisms, of which lactic acid bacteria are the most important, as, under ideal conditions, they rapidly make growth conditions inhibitory for other unwanted types of bacteria. The fermentation pattern is, however, not simple, even when only the lactic acid bacteria are considered. In silage two fermentative types of these organisms are always encountered. One is the homofermentative type, which, under anaerobic conditions forms approximately two moles of lactic acid per mole of glucose fermented.[11] The second is the heterofermentative type which produces, anerobically, one mole of lactic acid, one mole of CO_2 and one mole of ethanol per mole of glucose fermented.[11] On this evidence alone, it is clearly impossible to predict the final ratio of products of a lactic acid fermentation because a mixed population always develops and it is possible for a 100% variation to occur in the amount of lactic acid produced under two apparently similar circumstances.

The type of sugar, whether glucose or fructose, is also of importance (Table 2) as heterofermentative lactic acid bacteria ferment fructose and glucose by slightly different pathways; this results in different ratios of the final products from the two sugars with additional substances being produced from fructose, namely mannitol and acetic acid in place of ethanol. Less lactic acid is produced from fructose than from glucose, and consequently the efficiency of fermentation is considerably less than that of the homofermentative types of lactic acid bacteria.

From this simplified picture, it can be seen that fermentation can be grossly inefficient and could possibly explain why silages with apparently sufficient sugar content sometimes fail to preserve satisfactorily. The formation of mannitol, which has been shown to be present in silages, is seen to be a particularly wasteful process as its reduced nature makes it unavailable to many lactic acid bacteria. Obviously predicting fermentation products when considering sugars alone is a complex exercise. The picture is further confused, however, when the action of some of these lactic acid bacteria on plant non-nitrogenous organic acids is considered.

Organic acids (non-nitrogenous)

Considerable attention has recently been given to the specific organic acid content of plants and silage[1,2,12,13]. The organic acids and their salts form the important buffer systems in the plant[2,3]. Of the acids generally present, malate, citrate and phosphate are the most significant in a wide range of plant species. In silage, ~~one~~ of particular interest are those acids buffering within the range pH 6-4. The magnitude of the buffering action can be expressed as the buffering capacity determined by titration with either lactic or mineral acids. In a fresh grass crop, the buffering capacity is roughly equivalent to about 3% lactic acid in the dry matter, that is to say excluding any other changes which may occur, most grasses could be preserved at pH 4.0 by the addition of about 3% lactic acid on a dry matter basis[14]. Legumes, such as clover, usually contain higher amounts of organic acids than do grasses. In addition to the main organic acids present in grasses (malic and citric) clovers contain large amounts of glyceric acid[1,2]. Consequently, the buffering capacity of clovers proves to be about twice that of grasses, requiring about 6% lactic acid to bring the pH down to 4.0, on a dry weight basis[14]. This high buffering capacity and a generally lower sugar content than that found in grasses explains the difficulties often encountered in ensiling legumes successfully.

During ensilage marked changes in buffering capacity take place, resulting in an increase in the amount of acid required to lower the pH, over that required before ensilage[2,3]. In our experience this change can be explained to a large extent by the action of bacteria on the organic acids. Even under the best possible bacteriological conditions, i.e. a microflora dominated by a homolactic population of lactic acid bacteria, these changes still take place. Consequently, we need only consider the actions of lactic acid bacteria on malate and citrate to see how such increases in apparent buffering capacity take place. The increases can be up to 2 to 3 times that of the original plant material[2]. Obviously this markedly affects the original estimates of acid necessary for preservation.

Lactic acid bacteria of both the homo- and hetero-fermentative type will readily dissimilate malate and citrate by a number of pathways. (Table 3). The products formed are either neutral (acetoin, 2,3-butane diol and ethanol), salts of organic acids (lactates and acetates) or alkaline released cations. As many of the organic acids are present in the plant material in salt form, their destruction by bacteria acts against preservation, as decarboxylation results in the release of cations and carbon dioxide. The latter represents a gaseous loss, the cations neutralise the fermentation acids which buffer against a drop in pH. The overall effect is an apparent increase in buffering capacity during ensilage which cannot be avoided as even the most beneficial of the organisms present carry out these reactions. This situation coupled with the unpredictability of the course of sugar fermentation shows how difficult it is to draft a 'formula' for preservation based merely on carbohydrate content and 'assumed' buffering capacity of the crop. In order to make maximum use of the sugar available in the difficult crops, the addition of a sugar source or inoculum or both may be desirable. Alternatively short-term wilting will assist preservation as the sugar content will remain relatively stable while the loss of moisture will inhibit clostridia, so making a critical preserving pH easier to achieve. Some consideration has been given to the composition of an inoculum, and to the time it may best be added to the silage crop. Obviously the best type of lactic acid bacterium to add is a homofermentative type because of its economy of acid production. The choice of a particular species, however, has not proved to be so straight forward. At the present time an inoculum of Streptococcus faecalis or a mixture of S. faecalis and Lactobacillus plantarum seems to be the best choice. Results of laboratory studies have confirmed the advantages of adding a mixed inoculum to herbage at the time of ensiling. In tube-silo experiments in which a mixed inoculum of S. faecalis and L. plantarum (10^7 organisms/g fresh grass of a 1:1 mixture) was added to Italian ryegrass (dry matter 15.7%, WSC in dry matter 13.8%) it was demonstrated that the pH fell more rapidly than in the control silages. (Fig. I). A similar result was obtained in which an idential mixture (10^5 organism/g fresh grass) of bacteria were added to cocksfoot grass (dry matter 18.0%; WSC in dry matter 12.3%). In both cases the inoculated silages were well preserved compared with the control silages which were of pH values above 5.0. The practical application of these findings is at present being investigated.

S. faecalis grows under both aerobic and anaerobic conditions and can be added to the crop by a drip-inoculum system at the time of cutting. Although the organism is not acid tolerant, it reduces the pH rapidly to a level at which lactobacillus growth is favoured and the final fall in pH is accomplished by lactobacilli naturally resident on the crop. By adding an inoculum of L. plantarum as well, the last part of the acidification can be controlled. An inoculum of L. plantarum by itself has not proved to be ideal as initially it does not grow rapidly enough to dominate the anaerobic flora and ensure economy of the sugar in fermentation. If the crop is well lacerated and bruised, fermentation can be particularly rapid.

Amino Acids

The last aspect to be considered is the changes in the main nitrogenous components of silage. Such changes are important both from the question of losses and the possible effect upon the animals intake of dry matter.

Harvesting of a forage crop is followed by rapid and extensive proteolysis which is only terminated either by the attainment of a high dry matter or a low pH. Even in well preserved silage, about 50-60% of the protein is broken down. Since the fresh crop generally contains 80-90% of its nitrogen in the form of protein, large amounts of amino acids are released and it is of some importance to know their fate. In the silo the major changes to the amino acids are brought about by clostridia. The extent of clostridial activity is controlled as described earlier, by the water content and acidity of the material. A classical indication of clostridial action is the production of butyric acid by the saccharolytic vari~~es~~ ties from lactates and residual sugars (Table4). The destruction of two moles of lactate to yield one mole of butyric acid, carbon dioxide and hydrogen, results in a raising of pH towards neutrality. Such a change encourages the growth of the harmful putrefactive clostridia. A certain amount of putrifaction may also have occurred in silages which have an apparently satisfactory low pH. The likely explanation derived from the laboratory silo experiments described earlier, is that the pH drop has been relatively slow and clostridial activity has occurred in the early stages before being inhibited. Rate of pH fall, therefore, is an important feature of preservation.

Destruction of amino acids is by three main pathways[15] as shown in Table 4:-

(1) A coupled oxidative-reduction of a pair of amino acids resulting in the production of fatty acids, carbon dioxide and ammonia. (Stickland reaction).

(2) The fermentation of single amino acids to fatty acids, carbon dioxide and ammonia.

(3) The decarboxylation of amino acids with the formation of amines.

Decarboxylation products

The amines, cadaverine, putrescine, histamine, γ-amino butyric acid, B-alanine, tyramine and tryptamine have all been detected in silages[16,17,18]. These substances are of interest because of their possible effect upon the health of animals and there are various reports concerning histamine and tryptamine[18,19,20] particularly. The conditions in silage affecting amine formation, particularly cadaverine and putrescine have been studied in detail[17,21]. Their sources are lysine and arginine respectively. Conditions known to affect clostridial activity also control the formation of these substances. Most notable perhaps, has been the rate of pH fall during ensilage, rather than final pH value, upon the production of these amines[21]. As has been discussed before, it appears essential that to prevent clostridial activity a rapid drop in pH needs to be encouraged.

Deamination products

In this instance, the major products are fatty acids of a number of types, carbon dioxide and ammonia (Table 4). It is this activity, particularly, which results in marked nitrogen losses if clostridial activity is not controlled.

Finally it should be mentioned that bacteria other than clostridia are able to ferment amino acids. Lactic acid-forming bacteria, for instance, can deaminate both serine and arginine and decarboxylate other amino acids, so it is unlikely that perfect control will be obtained under natural conditions.

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TABLE I

The soluble carbohydrates of L. multiflorum (% dry matter)

Sample	Dry matter	Sucrose	Glucose	Fructose	Fructosans	Sol. carbohy. as monomers Fruct.	Gluc.	Ratio Fruct./Gluc.
1	19.2	3.5	2.2	1.9	5.6	10.0	4.1	2.4
2	19.9	4.5	1.6	1.3	7.0	11.5	4.0	2.9
3	14.9	3.2	1.7	1.0	9.5	13.3	3.4	3.9
4	21.5	8.4	2.2	1.9	5.9	11.0	6.6	1.7
5	15.6	2.4	2.3	3.8	6.0	11.8	3.6	3.3
							Mean	2.8

TABLE II

Main products of carbohydrate fermentation by lactic acid bacteria

HOMOFERMENTATIVE

(i) 1 Glucose ⟶ 2 Lactic acid

(ii) 1 Fructose ⟶ 2 Lactic acid

(iii) 1 Pentose ⟶ 1 Lactic acid + 1 Acetic acid

HETEROFERMENTATIVE

(i) 1 Glucose ⟶ 1 Lactic acid + 1 Ethanol + 1 CO_2 (if fermented)

(ii) 3 Fructose ⟶ 1 Lactic acid + 2 Mannitol + 1 Acetic acid + 1 CO_2

(iii) 1 Pentose ⟶ 1 Lactic acid + 1 Acetic acid

TABLE III

Main products of organic acid fermentation by lactic acid bacteria

HOMO- and HETERO-FERMENTATIVE

(i) 1 Citric acid $\longrightarrow$ 2 Acetic acid + 1 Formic acid + 1 CO_2

or

2 Citric acid $\longrightarrow$ 2 Acetic acid + 1 Acetoin + 4 CO_2

or

2 Citric acid $\longrightarrow$ 3 Acetic acid + 1 Lactic acid + 3 CO_2

(ii) 1 Malic acid $\longrightarrow$ 1 Lactic acid + 1 CO_2

or

2 Malic acid $\longrightarrow$ 1 Acetoin + 4 CO_2

or

1 Malic acid $\longrightarrow$ Acetic acid/Ethanol + Formic acid + CO_2

TABLE IV

Some examples of clostridial fermentation

Organic acids

2 Lactic acid $\longrightarrow$ Butyric acid + 2 CO_2 + 2H_2

Amino acids

(i) Coupled oxidation-reduction reactions (Stickland)

Alanine + 2 Glycine $\longrightarrow$ 3 Acetic acid + 3NH_3 + CO_2

(ii) Deamination

3 Alanine $\longrightarrow$ 2 Propionic acid + Acetic acid + 3 NH_3 + CO_2

Valine $\longrightarrow$ isobutyric acid + NH_3 + CO_2

Leucine $\longrightarrow$ isovaleric acid + NH_3 + CO_2

(iii) Decarboxylation

Histidine $\longrightarrow$ Histamine

Lysine $\longrightarrow$ Cadaverine

Arginine $\longrightarrow$ Ornithine $\longrightarrow$ Putrescine

Tryptophan $\longrightarrow$ Tryptamine

Tyrosine $\longrightarrow$ Tyramine

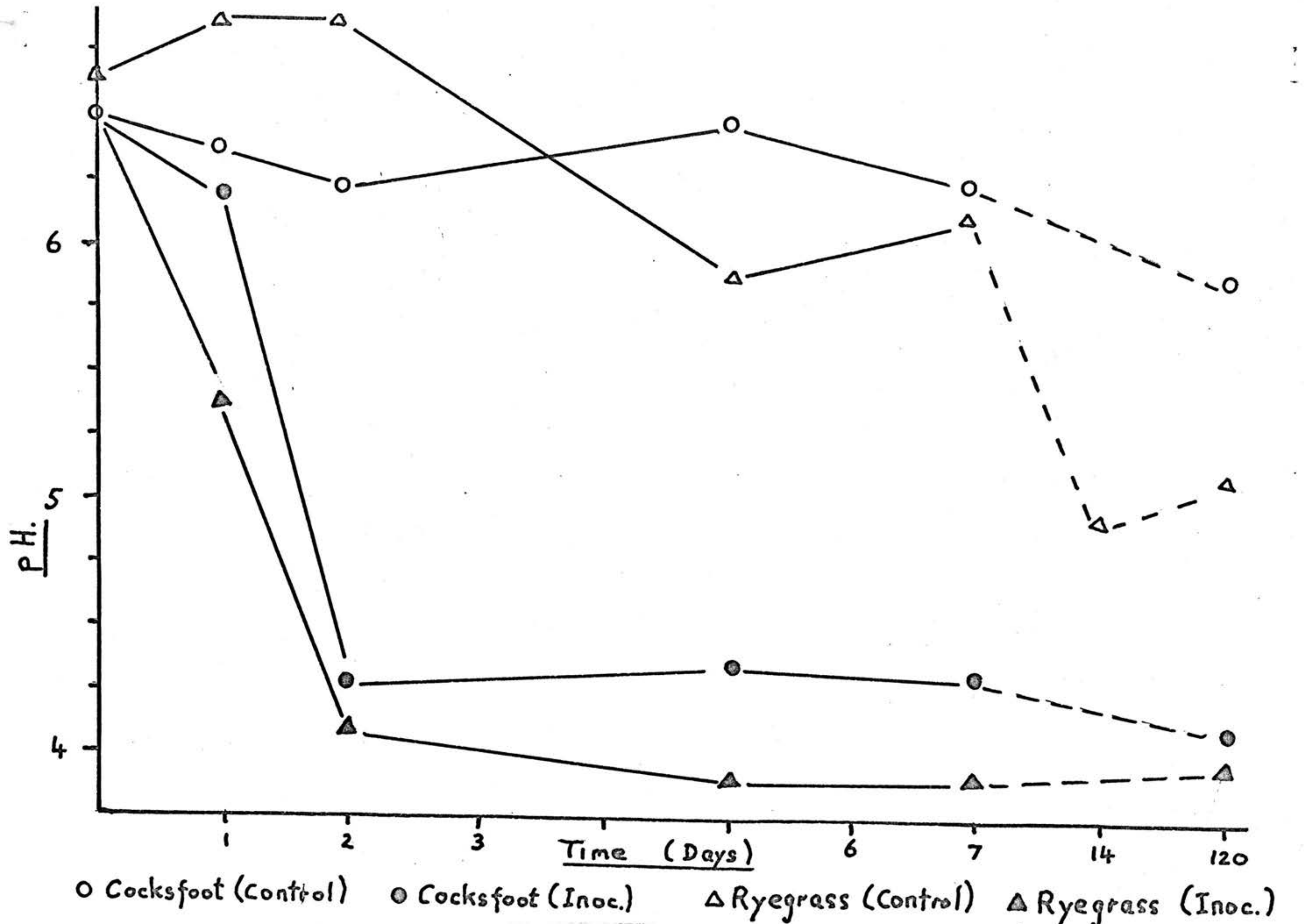

Fig I. Effect of inoculation of a 1:1 mixture of S. faecalis and L. plantarum on the pH of silages.

Confidential until publication.

LOSSES DURING ENSILAGE

by

P. McDonald and R. Whittenbury

SUMMARY

The magnitude and causes of losses of nutrients which occur during ensilage are discussed. From a consideration of the main biochemical pathways of the lactic acid bacteria, the losses which occur during fermentation in well preserved silages, should not exceed 5-6%. Where losses are high during ensilage, these can be attributed mainly to aerobic respiration.

INTRODUCTION

The preservation of a green crop by natural fermentation necessarily involves considerable biochemical changes which are accompanied by losses of nutrients. The most logical way of arresting these biochemical reactions is to inactivate the plant and microbial enzyme systems by the addition of inhibitors, and this is the object of using mineral acids as in the A.I.V. process. Unfortunately complete enzyme inactivation and sterilisation presents practical difficulties, and systems such as the A.I.V. process are not popular in the U.K.

Most silages in this country are made by the natural process which ~~depends~~ depend upon the formation of lactic acid from sugars in the herbage by the lactic acid bacteria and the subsequent preservation of the mass by maintaining a low pH value under anaerobic conditions.

A number of workers have reviewed the literature on losses during ensilage [23,24,25,30] and of these Watson & Nash[30] have made the most comprehensive study. From an examination of over 1,000 experiments these workers found that the dry matter losses for silages made from fresh herbage by the natural process averaged 17.3% and those from wilted herbage 13.4%. A study of the losses of digestible nutrients from a much smaller number of experiments indicated that losses of starch equivalent and digestible crude protein were approximately double the dry matter losses. Possibly the greater significance is the spread of these figures and losses of dry matter ranging from 2%[18] to over 40%[5] have been reported.

Before examining the nature of some of the high losses reported it is appropriate to consider some of the techniques used in measuring losses.

TECHNIQUES

Equipment:-

In order to study the accurate measurement of losses a number of workers have constructed experimental silos in which a regular record of weight change can be made[10,14,15]. Using silos of this type it has been possible to fractionate losses into those resulting from fermentation, surface waste and effluent. Some of the Edinburgh results are reported at the end of this paper.

Analytical errors:-

The importance of an accurate determination of dry matter has been stressed by many workers[6,16,29,30] and a method for correcting for volatile losses by analysis of fresh and oven dried samples was devised by Watson & Ferguson[29]. More recently more rapid methods based on the toluene distillation technique have been devised[2,11]. Using this technique, Minson & Lancaster[22] reported losses of dry matter on oven drying of from 2.7 to 16.6% while Wilson et al[31] have reported dry matter losses of up to 22.1% during forced-air oven drying.

NATURE OF LOSSES

The losses which occur during ensilage can be considered under four main headings:- 1. Field losses, 2. Respiration (aerobic) losses in the silo, 3. Fermentation (anaerobic) losses, 4. Effluent losses.

Field losses:-

With crops cut and ensiled the same day, losses of dry matter are considered to be negligible. Over a 24 hour wilting period losses of not more than 1 or 2% of dry matter can be expected. In some experiments gains in dry matter have in fact been reported,[25] and it has been postulated that these could arise from photosynthesis. That photosynthesis does occur in the cut plant, even upto 4 days after cutting has been proved from radioisotope studies using $[C^{14}]O_2$[20]. It is doubtful however if in most instances the synthesis of sugars can compete with those lost through respiration. Over periods of wilting longer than 48 hours, considerable losses of dry matter can occur depending upon the drying conditions. Dijkstra & Brandsma[4] have reported losses of dry matter of 6.4% after 5 days and 10.4% after 8 days.

Respiration losses in the silo:-

The activity of the plant enzymes will continue in the silo as long as conditions are aerobic and the pH is not drastically changed. The increase in temperature which occurs in the silage mass depends upon the rate of respiration, degree of insulation of the silo and the specific heat of the ensiled material. The rate of respiration is controlled by the temperature. Within the temperature range 0-30°, the acceleration of respiration with temperature is roughly exponential.[8] Above 30°, the rate of increase per unit of temperature slowly decreases but rise in temperature still accelerates respiration until the enzymes are inactivated. The specific heat of dried grass has been estimated to be about 0.45 and it follows therefore that under aerobic conditions the drier the herbage, the more rapid the temperature increase. One of the main objects in ensilage is to maintain anaerobic conditions by preventing repenetration of air. In a sealed silo, the oxygen trapped within the herbage is of little significance and will only bring about a temperature increase of 3 or 4 degrees with the oxidation of not more than 1% sugar in the dry matter[19]. It is clear therefore that if losses are to be kept low it is necessary to achieve anaerobic conditions as rapidly as possible.

In most farm silos considerable aerobic respiration resulting from both plant and microbial enzymes takes place on the surface and sides of the silage. It is often difficult to separate and assess the extent of this waste material and in many experiments in which losses have been measured the 'waste' material has been separated visually from the 'well preserved' silage and weighed. The weight of this material is then expressed either as a percentage of the total silage removed or of the total weight of herbage originally ensiled. Unfortunately it is difficult to assess the dry matter losses which have occurred in the production of this waste material and very often these losses are erroneously attributed to fermentation losses. In order to assess the true losses in the formation of waste it is necessary to know the original weight of herbage from which the waste material was produced. Kennedy and co-workers[12] have measured these surface losses by using a bag and marker technique. A modification of this technique using 'Terylene' netting as a marker sheet and perforated polythene bags has been described by the authors in an earlier paper[17]. In our studies using this technique, we have found that upto 73% of the original herbage dry matter can be lost in the production of high pH waste material.

Fermentation losses:-

Some confusion in the past has arisen over the use of the term 'fermentation', and in many studies the difference between the total dry matter loss from the silo and dry matter lost, via the effluent has been described as 'fermentation loss'. This is a grossly incorrect assumption since a major part of the loss attributed to fermentation does in fact result from respiration. Fermentation losses, in the strict sence, are those arising from the activities of micro-organisms under anaerobic conditions. If this is accepted then it should be possible to predict the fermentation losses from a knowledge of the biochemical pathways of the micro-organisms involved.

In well preserved non-wilted grass silage, the dominant organisms will be lactic acid bacteria. These can be classified into two main types - (1) the homolactic organisms, and (2) the heterolactic organisms. The former are more efficient at producing lactic acid from hexoses in that 2 moles of lactic acid are produced from 1 mole hexose without any gaseous products. In a completely homolactic type of fermentation therefore, the gaseous losses during the production of lactic acid from sugars will be nil.

In many silages, however, the heterolactic organisms dominate the flora and the products vary depending upon whether glucose or fructose are fermented (<u>Table I</u>). The final column in Table I shows the dry matter loss as a percentage of the nutrient fermented. The fermentation pathways with hexoses are fairly straightforward but with organic acids several different pathways are possible. In the case of amino acids, present evidence suggests that only serine and arginine are significantly attacked by the lactic acid bacteria and the gaseous loss from the silo will depend to a large extent upon whether or not the free ammonia formed is volatile or is fixed by neutralisation with organic acids. In well preserved silage, of low pH value, there is likely to be little loss of ammonia.

Taking a typical sample of Italian ryegrass of the composition as shown in Table 2, (for this purpose fructosans and sucrose have been expressed in terms of their monomers) and assuming a heterolactic fermentation of the least efficient type, then fermentation of the hexoses, organic acids and amino acids will result in a gaseous loss of about 4%. These calculations do not take into account any possible loss from subsequent degradation of hemicelluloses. Pentoses may evenually become available as a result of acid hydrolysis of hemicelluloses[2], and these will be fermented as follows:-

1 Pentose ⟶ 1 Acetic acid + 1 Lactic acid

In this pathway, carbon dioxide is not produced, so that pentose fermentation does not increase the gaseous loss figure. Also shown in Table 2 is the amount of lactic acid produced from these reactions. Results from our experiments with Italian ryegrass suggest that the lactic acid produced from a crop containing 15% water soluble carbohydrates would in fact be higher than 5.4% as shown in Table 4, indicating that in practice some homolactic fermentation would normally occur. A fermentation gaseous loss of 4% can therefore be regarded as a maximum value for this type of herbage.

It can be seen from the pathways shown in Table I that the highest dry matter losses occur during the fermentation of organic acids. Since these pathways are similar for both homo- and hetero-lactic types of organisms, the organic acid content of plants assumes considerable importance in silage making.

The organic acids in the dry matter vary from 2 to 6% for grasses and 6 to 8% for legumes[7] and clearly at the higher levels the dry matter losses during ensilage could be higher than those calculated in the example. In the case of clovers, glyceric acid has been found to be the major acid[13,26] and although this disappears during ensilage, the exact fermentation pathways have not yet been elucidated.

As far as fermentation by heterolactic bacteria are concerned it would appear therefore that the maximum losses of dry matter should not exceed 4-5%. In practice the figure might be rather less than this because of some homolactic fermentation of the carbohydrate fraction.

A further factor which might reduce this figure is the possibility of fixation of carbon dioxide, and, there is some evidence for this from experiments using ^{14}C-labelled CO_2[12].

If a clostridial fermentation occurs, then the losses are likely to be higher than those calculated above. Clostridia are inhibited either by dry conditions or by a high hydrogen ion concentration. Wet crops of low soluble carbohydrate content, are ideal for clostridial growth. The products of clostridial metabolism are many and varied. A few of these are shown in Table 3. It is difficult to consider all the possible dry matter losses involved in a clostridial type fermentation because of the variety of reactions. Inevitably because of lactic acid breakdown and deamination or decarboxylation of amino acids, the losses will be much greater than those occurring in a lactic acid type fermentation.

Finally the fermentation losses will be governed by the dry matter content of the ensiled crop. If the crop has been wilted then fermentation will be inhibited at a higher pH value than if a fresh crop had been ensiled. This is well illustrated from the results of laboratory studies shown in Table 4, in which silages were made from Italian ryegrass after different degrees of wilting.

Losses in the effluent:-

The volume of effluent produced from a silo is dependent upon several factors of which the most important is the moisture content of the crop. Miller & Clifton[21] studied the effluent production from 62 silos and calculated the following prediction equation:-

$D = 17.614 - 0.538X$ where $D =$ % dry matter lost and $X =$ % dry matter of the ensiled material. From this equation, the dry matter content of the crop at zero effluent production would be 32.7%. In a different study, Sutter[28] obtained a regression equation relating volume of effluent produced to the dry matter of the crop:-

$V = 66.94 - 2.24X$ where $V =$ litres of effluent/100 kg silage and $X =$ % dry matter of the ensiled crop. From Sutter's equation zero effluent production is at 30% dry matter. Sutter predicted volumes of effluent are in good agreement with the experimental results of Jones & Murdoch[9]. It has been shown that consolidation pressure can influence effluent production[15] and our studies indicate that size and type of silo may also be important. The dry matter content of the effluent has been found by various authors to range from 2 to 10% and to be frequently about 6%[23]. There is a variation in the composition from any one silo in that % dry matter and % nitrogenous compounds increase, while sugar concentration decreases[15]. In addition to a loss of soluble organic compounds, the effluent is a valuable source of mineral elements[27].

CONCLUSIONS

The results of our investigations and those of other workers indicate that where high losses occur during ensilage, these are mainly due to aerobic respiration and not fermentation as has sometimes been suggested. The detailed losses from a number of experiments carried out by the authors are summarised in Table 5. In these studies the waste losses have been measured using the bag and marker technique described earlier. The relatively low fermentation losses of these well preserved silages are in reasonable agreement with the biochemical calculations made earlier. Our results also confirm the general principle of silage making that in order to keep the losses down to a minimum then crops of a dry mattercontent above about 30%, should be ensiled. If anaerobic conditions are obtained rapidly, and maintained throughout the storage period, then the only losses encountered will be those resulting from fermentation and a small amount of respiration, which in a lactic acid type fermentation should not exceed 4-6% of the dry matter.

In practice the vacuum silo approaches the ideal, although the vacuum itself is probably of little significance, and the main advantage of this system is that, if properly carried out, anaerobic conditions are maintained throughout the storage period and surface waste is eliminated.

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TABLE 1.

Some fermentation pathways of heterolactic bacteria

		D.M. loss (% of nutrient)
Carbohydrates		
Glucose $\longrightarrow$	Lactic acid + Ethanol + CO_2	24
3 Fructose $\longrightarrow$	Lactic acid + Acetic acid + 2 Mannitol + CO_2	5
Organic acids		
Malic acid $\longrightarrow$	Lactic acid + CO_2	33
or 2 Malic acid $\longrightarrow$	Acetoin + 4 CO_2	67
or Malic acid $\longrightarrow$	Acetic acid + Formic acid + CO_2	21
2 Citric acid $\longrightarrow$	2 Acetic acid + Acetoin + 4 CO_2	46
or Citric acid $\longrightarrow$	2 Acetic acid + Formic acid + CO_2	14
or 2 Citric acid $\longrightarrow$	3 Acetic acid + 1 Lactic acid + 3 CO_2	30
Amino acids		
2 Serine $\longrightarrow$	Acetoin + 2 CO_2 + 2 NH_3	42 (58)*
Arginine $\longrightarrow$	Ornithine + CO_2 + 2 NH_3	5 (24)*

*If NH_3 lost

TABLE 2.

Theoretical calculation of maximum losses during fermentation of major nutrients of Italian ryegrass, assuming a heterolactic fermentation

Composition of grass (% D.M.)		Nutrient lost (%)	Total D.M. lost (%)	Lactic acid formed (%) (maximum)
Fructose	10	5	0.5	1.7
Glucose	5	24	1.2	2.5
Malic acid	1.5	67	1.0	1.0
Citric acid	1.0	46	0.5	0.2
Serine	0.8	58	0.5	-
Arginine	1.1	24	0.3	-
(Crude Protein	15)			
Total			4.0	5.4

TABLE 3.

Some products of clostridial fermentation

Organic acids

2 Lactic acid $\longrightarrow$ Butyric acid + 2 CO_2 + 2 H_2

Amino acids

(1) Energy yielding pathways

3 Alanine $\longrightarrow$ 2 Propionic acid + Acetic acid + 3 NH_3 + CO_2

or

Alanine + 2 Glycine $\longrightarrow$ 3 Acetic acid + 3 NH_3 + CO_2

(ii) Decarboxylation

Histidine $\longrightarrow$ Histamine + CO_2

Lysine $\longrightarrow$ Cadavarine + CO_2

(iii) Deamination

Valine $\longrightarrow$ isobutyric acid + NH_3

Leucine $\longrightarrow$ isovaleric acid + NH_3

TABLE 4.

Relationship between dry matter and silage composition

Sample	Dry matter	pH	Buffer capacity	Water soluble carbohydrates
	%		m-equiv/100 g. d.m.	% d.m.
Fresh grass	15.5	6.1	43	10.9
Silage 1.	15.5	4.2	81	5.3
Silage 2.	23.0	4.4	74	6.2
Silage 3.	36.6	5.0	60	7.0
Silage 4.	42.1	5.1	54	9.1
Silage 5.	55.8	6.3	47	9.7

TABLE 5.

Fractioned losses of dry matter during ensilage of unwilted grasses and clovers

(Values expressed as % dry matter ensiled)

	Grasses			Clovers		
	Range	Mean	S.D.	Range	Mean	S.D.
(a) Total edible loss	5.9–23.6	12.0	±5.91	17.0–26.1	20.8	±3.48
(b) Total loss from silo	2.9–16.2	9.5	±4.30	9.2–21.1	14.3	±3.85
(c) Surface waste	0.6– 3.9	2.0	±0.99	2.5– 5.1	3.6	±0.92
Fermentation loss	+3.6– 7.4	3.1	±3.33	+ 3.1– 9.9	3.8	±4.61
Effluent loss	0–11.6	4.4	±4.34	2.6–14.0	6.9	±3.96
Number of samples		14			8	

(a) Including weight of waste

(b) Excluding weight of waste

(c) Gaseous loss only

Chemical changes and losses during the Ensilage of Wilted Grass

by

P. McDonald, A. R. Henderson and A. W. MacGregor

Summary

Two experiments were carried out; in the first wilted Italian ryegrass, at two different dry matter (DM) levels (34% and 47%) was ensiled. In the second, fresh grass (15.9% DM) as well as similar herbage wilted to 30.3% DM were ensiled. Total edible DM losses from the wilted silages were low ranging from 6.7 to 10.4. Changes in individual sugars and organic acids were followed. The residual amounts of sugars in the wilted silages were directly related to the degree of wilting. All silages were well preserved, but little fermentation had occurred in the 47% DM wilted material. From a knowledge of the sugars lost and amounts of mannitol and ethanol formed it has been possible to examine quantitatively the main biochemical ~~reactions~~ changes during the ensilage of the wilted materials. The results confirm the efficiency ~~by~~ with which wilted grass is anaerobically conserved by the fermentation products of lactic acid bacteria.

Introduction

The advantages of wilting crops, prior to ensiling, to a dry matter content of 30% or above have been stressed by many workers[1-5]. Apart from reducing or eliminating effluent losses, the reduction in moisture content discourages clostridial activity and produces a silage which is more acceptable to ruminant animals[6]. It has been shown that high dry matter silages have higher pH values and contain~~s~~ more residual sugars than unwilted silages. Studies of losses during ensilage of wilted grass have given variable results although most workers agree that provided the silo is adequately sealed, dry matter losses are low.

In spite of the well established advantages of wilting, there is still a lack of information about the detailed reactions which take place during the fermentation of such crops and the purpose of this investigation was to carry out a comprehensive study of the chemical changes which occurred during ensilage of partially wilted grass, and herbage wilted to a high dry matter content.

Experimental

Two separate experiments were carried out. In the first wilted herbage, at two different dry matter levels (34% and 47%) was ensiled. In the second experiment, fresh grass (15.9% dry matter) as well as similar herbage wilted to 30.3% dry matter were examined.

Experiment 1.

Procedure

The silo unit used in this study has been described in an earlier publication[8], and consists of 4 metal silos, each having a maximum capacity of approximately 1000 kg fresh herbage. In this experiment conditions of filling were similar to those described in previous publications[8,9]. Consolidation and sealing ~~was~~ were achieved by covering the ensiled herbage with polythene sheeting and stone blocks corresponding to a surface pressure of 37 g/sq. cm. Assessment of true losses and surface waste measurements were made using a bag and marker technique described in an earlier paper[9].

Italian ryegrass (Lolium multiflorum) obtained from one of the School farms was cut with a mower/crimper on 31st May, 1965 and wilted for 29 hours in the field before being lifted with a flail-type forage harvester and ensiling in silos A and B. During this period the grass was tedded 3 times. Similar material was tedded twice during a further 23 hours wilting period before being lifted with a forage harvester and ensiled in sølos C and D. The weather was fine and sunny throughout the 52 h. wilting period.

A total of 788 kg of wilted herbage was ensiled in each silo, the dry matter contents of the wilted materials were 34.0% and 47.0% for the 29 h.and 52 h. wilted grasses respectively. The silos were opened 57 days after filling. Weights (kg) of silage removed were:- A, 779, B, 776, C, 778, D, 777. In order to obtain a measure of losses during wilting in the field, six areas per treatment, each 6' x 5' were weighed and sampled before and after wilting on 'terylene' netting placed at random in different parts of the field. During this period, field mechanical treatments were simulated by hand on each of the nets.

Laboratory silos

In addition to filling the 4 large silos, similar herbage was used to fill tube silos (32 mm x 200 mm) of 70 g capacity for detailed chemical studies in the laboratory. These tubes, which were kept at room temperature (C.20^{o}) in the dark, were opened and the contents examined after 30 h. 14 and 38 days.

Analytical and digestibility techniques

Individual sugars were determined using paper chromatographic techniques, using ethyl acetate : pyridine:water (10:4:3), and ethyl acetate:acetic acid: formic acid:water (18:3:1:4) solvent systems, and ethanol by the method of Kent-Jones and Taylor[10]. Organic acids were determined by column chromatography[11,12]. Acetoin, diacetyl and 2,3-butanediol by standard methods[13]. A toluene distillation procedure was used for estimation of dry matter[14].

Mannitol was isolated by paper chromatography using the above solvent systems and converted by meta periodate oxidation to formic acid according to the method of Hirst and Jones[15]; the formic acid was then determined by the procedure of Kolthoff and Belcher[16]. // Digestibility trials were carried out in triplicate on grass and in duplicate on the four silages using

Cheviot wether sheep. During these trials, the animals were fed to appetite.

Experiment 2.

In the second experiment carried out in May 1966, an attempt was made to reduce oxidative losses to a minimum by sealing the silos with heavy gauge polythene. In this case, consolidation weights were not applied and the plastic top was bonded with adhesive to the outer flange of the silos. Only two silos were used, the first (silo A) was filled with 1045 kg of fresh Italian ryegrass cut with a flail-type forage harvester; the dry matter content of this herbage was 15.9%. The second (silo B) was filled with 1045 kg of similar material wilted in the field for 52 hours (dry matter 30.3%). During the period of wilting, weather conditions were poor and showers fell at intermittent intervals. Thermocouples and marker sheets were not used in this experiment. The silos were opened after 141 days and weights of silage removed were A, 882 kg, and B, 1034 kg.

Effluent (total 153 kg) was obtained only from silo A and this was collected daily or when it appeared, and stored at -18° until subsequently analysed. Field losses and digestibility measurements were not made in this experiment.

A number of laboratory tube silos were, however, filled with fresh and wilted herbage and these were opened at various intervals of time during the period of the experiment.

Results

Experiment 1.

Temperature changes

The temperatures in the four silos remained relatively low throughout the period of ensilage. Maximum values, which occurred on the 10th day, in the surface layers were (A) 23°; (B) 21°; (C) 24° and (D) 28°. This corresponded to a high ambient air temperature (20°) on the previous day.

Composition

The composition of the wilted grasses and silages is shown in Table 1. The main changes which occurred affected the nitrogen and soluble carbohydrate fractions. With regard to the former, considerable proteolysis had clearly occurred, although the very small amounts of volatile N indicated that little deamination had taken place. About 50% of the original water soluble carbohydrates (WSC) were recovered in the 34% dry matter silages whereas in the higher dry matter (47%) silages about 75% of the WSC in silage C and 95% in silage D were recovered. These recovery figures are, however, slightly misleading when based on total WSC figures because of the presence of pentoses in the silages which had been released during ensilage from hemicelluloses. Complete hydrolysis of fructosans and sucrose had taken place and this breakdown is reflected in the higher fructose values which were obtained for the silages.

A detailed organic acid analysis showed that the only volatile fatty acids present in the silages were acetic and propionic. Butyric, isobutyric, isovaleric and caproic acids, indicators of clostridial activity, were not detected in any of the silage samples.

Losses

The dry matter losses during wilting in the field were calculated from the 'terylene' net results, these averaged 1.4% for the 29 h. wilted grass and 4.0% for the 52 h. wilted material. These findings are in agreement with the general conclusions of other workers[17].

Dry matter losses during ensilage have been calculated in two ways; the gross or 'edible' loss includes the weight of waste and the 'loss from the silo' which excludes the weight of waste and consists solely of gaseous loss. (In the second experiment this last includes the weight of effluent). The 'losses from the silo' in experiment 1 were extremely small, averaging only 2.8% for silos A and B and 1.6% for silos C and D. The individual results are given in Table II. The weights of waste materials expressed in kg dry matter were - A, 13.0; B, 18.0; C, 14.5; and D 20.5. In order to avoid complications resulting from the production of this waste material in the surface layers, losses of dry matter and total hexoses were calculated together with the weights of the two alcohols, mannitol and ethanol in herbage and silage below the marker sheets which were sited in the silos when they were three-quarters full of herbage. These figures are given in Table III.

Digestibility and intake studies

The results of the feeding trials with sheep are given in Table IV. There was very little difference in digestibility between the grasses and silages although the 52 h. wilted materials tended to be of slightly lower digestibility than the 29 h. wilted foods. These differences were also reflected in the intake figures obtained for the grasses.

Laboratory silos

Laboratory silos were opened after 30 h., 14 and 58 days and analysed for soluble carbohydrates and organic acids. Results are shown in Table V. In addition to ensiling the two wilted grasses, the fresh herbage (dry matter 20.3%) was also examined. Rate of fermentation, assessed on the basis of sugar breakdown and acid formation, was clearly most rapid in the fresh material. In the 52 h. wilted material, very little change occurred after 14 days apart from some hemicellulose hydrolysis and some increase in acetic and lactic acids. The results indicate the facility with which fructosans and sucrose are hydrolysed. After 30 h., free fructose had increased in all silages, but whereas the fructose concentration was maintained in the wilted silages, the content of this sugar decreased in the fresh material during the ensiling period. The residual sugars in the silages after 58 days did not differ markedly from those found in similar material obtained from the large silos. The lactic acid content of the 29 h. wilted silage was, however, noticeably higher than that obtained in silage from the large silos.

Experiment 2.

Large silos

The method of sealing appeared to be successful in the case of silo B where no waste material was observed although the relatively high gaseous loss of dry matter (7.5%) suggested that some oxidation had occurred. In A, 28.4 kg of waste was produced which positively indicated that some air had gained entry after filling. The composition of the fresh and wilted grasses and silages are shown in Table 1. The WSC content of the original herbage was high (23.8%) and this was not markedly affected by wilting. In spite of this initially high level of sugars in the fresh crop, virtually all of this had disappeared during ensilage compared with a residual amount of 9.3% in the wilted silage. The lactic, acetic acid and pH values of the 28% dry matter silage were almost identical to the values obtained for the 34% dry matter silage in the first experiment, whereas the lactic acid content of the fresh silage was markedly higher (12.1%). Total edible loss of dry matter during ensilage of the unwilted grass (18.2%) was more than double that from the wilted material (7.5%) and, of this relatively high loss, 6.4% was attributable to the effluent. The latter contained large amounts of WSC (17.5% in the dry matter) and lactic acid (26.6%).

Laboratory silos

In this experiment, mannitol was determined in the tube silages in addition to individual sugars as is shown in Table VI. The total sugars in the samples of herbage taken from the tube silos were higher than those determined for the materials ensiled in the large silos. Mannitol was detected in measurable quantities after 48 h., and during this period of time in the unwilted herbage, sucrose had completely disappeared. Galactose was found to be present after 24 hours and this was presumed to have been formed from hemicellulase activity. Lactic acid was present in only trace~~s~~ amounts after 24 h. in both silages but after 48 h. was present in measurable amounts.

Discussion

It is well established that the extent of fermentation during ensilage is governed by the dry matter content of the crop[1,5,6,17], and this conclusion is also confirmed in these studies. In unwilted silages, there is very little residual soluble carbohydrate remaining and the composition of the wet silage A in the second experiment is typical of such material, in spite of an exceptionally initial high WSC content (23.8%) in the dry matter. On the other hand the residual soluble carbohydrates in the wilted silages were directly related to the dry matter content of the ensiled material. Unfortunately the significance of this to the animal in terms of productive performance was not adequately measured and clearly requires further investigation with regard to such factors as VFA pattern and utilisation of ME. There is some evidence, however, from previous studies that the presence of soluble sugars in silage may result in a more efficient utilisation of the nitrogenous components.

The major nutrients fermented during ensilage of grass by lactic acid bacteria are glucose, fructose and the organic acids, malic and citric. The fermentation products resulting from the breakdown of the major hexoses in grass vary depending upon whether the organisms responsible are of the homolactic or heterolactic type. The various pathways (excepting No. 5) have been reviewed in a recent publication[18] and these are summarised in Table VII. It can be seen that in the case of the homolactic organisms, lactic acid alone is normally formed whereas with the heterolactic bacteria, mannitol is a major product of fructose fermentation and ethanol of glucose fermentation. Since accurate weights of herbage ensiled and silage removed were recorded, it has been possible to calculate the actual amounts of fructose and glucose which have disappeared during ensilage. This has been done in Experiment I where, because of the marker and bag technique used, it has been possible to calculate the losses of nutrients below the marker sheet, thereby eliminating any errors caused by possible oxidation in the surface layers. In this exercise the amounts of mannitol and ethanol formed were used as a basis for the calculations involving pathways 3 and 4 given in Table VII. It became clear from the results obtained that in order to account for the relatively large amount of mannitol formed, the quantity of fructose required was greatly in excess of that actually available. Clearly an alternative pathway was indicated. Concomitant studies in the laboratory with isotopically [C^{14}] labelled hexoses indicated that a reaction involving a fermentation of glucose coupled with a reduction of fructose was possible according to equation No. 5 in Table VII[19]. This pathway has been used in the calculations which are summarised in Table VIII giving the weights of lactic and acetic acids together with carbon dioxide production. Also shown in the table are the calculated weights of acids and CO_2 derived from the fermentation of citrate and malate according to the reactions outlined in Table VII. It is known that there are alternative pathways[18] for the dissimilation of these two acids, but as the end products include acetoin, diacetyl, 2-3, butanediol, formic acid, and as none of these products were detected in the silages in Experiment 1 it has been concluded that the pathways shown in Table VII ~~were~~ are correct.

In these calculations the activities of the plant enzymes have not been taken into account. In the case of the sugars, it is unlikely that any marked respiration would have occurred since the quantity of oxygen trapped in the mass has been calculated to be extremely small and would not account for a hexose loss of greater than 0.5 kg. This can be compared with the total hexose loss of 33 kg in silo A for example.

The presence of small amounts of xylose and galactose in the silages indicated that some hemicellulose breakdown had taken place. Previous studies[20] with unwilted herbage, have shown that hemicelluloses disappear during ensilage to the extent of 11-55%, and that individual losses of polymers were preferentially, araban $>$ galactan $>$ xylan. These studies also showed that pentoses were produced by both the action of plant hemicelluloses and acid hydrolysis. Both homo- and hetero-lactic bacteria ferment pentose ~~similarly~~ according to the equation:- pentose $\rightarrow$ lactic acid + acetic acid.

It is perhaps relevant to note here that in silos A and B in Experiment 1, rather more lactic and acetic acids were in fact produced than was calculated by the reactions given in Table VII indicating another source of these acids. This source could have been free pentoses.

In the case of silos C and D of Experiment 1, the reverse is true for lactic acid where the calculated values were rather higher than the amounts actually found. The activities of bacteria in these dry silages ~~was~~ were clearly restricted, particularly in silo D, but it is possible that some yeast growth took place resulting in ethanol formation from hexoses. It is known that in wet herbage, bacteria are more active than yeasts which are normally present in such material in relatively small numbers[21]. On the other hand it has been established that in the ensilage of moist barley with dry matter contents as low as 60%, yeast organisms make the major contribution to the products of fermentation.

Although malic and citric acids are present in relatively small amounts in ryegrass compared to soluble carbohydrates, they nevertheless can make an important contribution to the lactic acid content of the silage. Malate can be degraded to a small extent by the action of plant enzymes, with the production of succinate, but it has been shown that the main breakdown of this acid is bacterial[22]. Succinate, although absent from the original grass, was found in small amounts in the silages in Experiment 1, it is difficult to conclude however, whether the presence of the acid was at the expense of malate.

Carbon dioxide production has also been calculated in Experiment 1, from the reactions given in Table VII. Expressed as a percentage of dry matter ensiled (below the marker sheet), CO_2 production was A, 1.9%; B, 1.8%; C, 1.4%; D, 1.1%. The actual dry matter losses during the ensilage of material below the marker sheet were A, 0.1%; B, 1.4%; C, 0.2%; D, +0.8%. Since these figures are so low, it may be unwise to attach significance to the differences although the possibility of CO_2 fixation can be considered. That this can take place during ensilage has recently been shown in studies in this laboratory[23] using $[^{14}C]$ labelled CO_2 where the main products of fixation were lactic and succinic acids.

In the second experiment, marker sheets were not used, since it was hoped to achieve completely air-tight conditions by sealing the silos with plastic. This was, however, not entirely successful and consequently it is difficult to evaluate the fermentation changes adequately. Silage B was very similar in composition to the silages made from the 29 h. wilted herbage in Experiment 1 and this suggests that a similar type of fermentation had occurred. The situation in silo A is complicated by the production of effluent, which contained high levels of both lactic acid and WSC. A further complication is indicated by the presence of formic acid and 2,3-butanediol which could have been formed from malate by phosphoroclastic cleavage. The presence of propionic acid in silage A, as well as in the silages of the first experiment, is worthy of comment. This acid could have resulted from either the coupled oxidation-reduction reaction (Stickland) of amino acids, deamination of alanine, or the action of propionibacteria on ~~pyruvate~~ sugars or lactate, reactions all of which result in the formation of acetic acid. In conclusion the results of these experiments show that the ensilage of wilted ryegrass under anaerobic conditions results in very little loss of nutrients and confirms that lactic acid bacterial fermentation is an efficient conservation process. As stated previously, where high losses occur during ensilage of wilted grass, these losses arise from oxidation and not from fermentation.

These results also suggest that there is little, if any, advantage to be gained in terms of reduced dry matter loss by wilting crops to dry matter contents of greater than 30-34%. Indeed, the higher dry matter losses in the field associated with prolonged wilting, may result in an higher overall loss. Whether the decreased extent of fermentation, with consequent saving in sugars, is of value to the animal, remains to be proven.

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TABLE I

Composition of grass and silages

(% of true dry matter)

	Experiment 1						Experiment 2				
	Grass		Silages				Grass		Silages		Effluent
	AB	CD	A	B	C	D	A	B	A	B	A
Dry matter	33.98	46.99	34.07	33.64	46.94	47.55	15.90	30.34	15.91	28.35	7.00
Organic matter	91.7	91.2	91.4	91.5	90.8	91.0	90.7	90.9	89.8	89.9	84.4
Crude protein	11.8	12.4	12.1	12.6	13.0	12.8	18.1	18.2	18.0	18.4	26.6
Ether extract	2.0	2.1	3.1	3.0	3.0	2.9	2.7	2.5	3.1	3.5	-
Crude fibre	24.6	24.5	25.6	25.8	26.1	26.0	19.2	20.2	22.0	22.0	-
Total N	1.89	1.98	1.94	2.02	2.08	2.04	2.90	2.91	2.89	2.95	4.25
Protein N	1.50	1.51	0.72	0.76	1.03	0.79	2.39	2.26	0.92	0.99	-
Non-protein N	0.39	0.47	1.22	1.26	1.15	1.14	0.51	0.65	1.97	1.93	-
Volatile N	0.02	0.02	0.12	0.12	0.09	0.08	0.01	0.03	0.20	0.19	-
Water-soluble carbohydrates	21.4	21.1	10.6	11.7	16.4	20.3	23.8	21.8	1.7	9.3	17.5
Glucose	4.4	4.2	2.5	2.4	4.5	5.3	*8.8	*8.2	nil	2.2	2.8
Fructose	4.2	4.2	6.3	6.4	9.1	11.6	*15.0	*16.6	nil	5.3	5.3
Xylose	nil	nil	0.4	0.5	0.4	0.5	nil	-	nil	0.6	nil
Galactose	nil	nil	0.7	0.6	0.5	0.6	-	-	nil	0.6	0.14
Sucrose	3.2	2.0	nil	nil	nil	nil	-	-	nil	nil	nil
Fructosans	9.1	8.7	nil	nil	nil	nil	-	-	0.9	0.6	4.3
Mannitol	nil	nil	7.9	7.4	3.9	3.2	nil	nil	5.6	6.8	10.9
Cellulose	27.1	26.8	28.0	28.7	28.1	28.1	21.9	22.8	24.7	24.4	-
Lignin	4.2	4.2	4.3	4.4	4.6	4.8	2.8	3.3	4.1	4.3	-
Formic acid	nil	nil	nil	nil	nil	nil	nil	nil	0.05	nil	nil
Acetic acid	nil	nil	2.13	2.06	1.15	0.68	nil	nil	3.62	2.10	6.26
Propionic acid	nil	nil	0.15	0.15	0.11	0.10	nil	nil	0.13	nil	nil
Butyric acid	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil	nil
Lactic acid	nil	nil	5.45	5.40	1.71	0.93	nil	nil	12.06	5.52	26.55
Succinic acid	nil	nil	0.18	0.19	0.13	0.08	Tr.	nil	nil	nil	nil
Citric acid	0.53	0.51	nil	nil	nil	nil	0.65	0.77	nil	nil	-
Malic acid	1.15	1.18	nil	nil	nil	nil	1.75	1.71	nil	nil	-
Fumaric acid	nil	nil	nil	nil	nil	nil	0.19	0.09	nil	nil	-
Malonic acid	nil	nil	nil	nil	nil	nil	0.13	0.13	nil	nil	-

TABLE I continued.

	Experiment 1						Experiment 2				
	Grass		Silages				Grass		Silages		Effluent
	AB	CD	A	B	C	D	A	B	A	B	A
Ethanol	-	-	0.45	0.35	0.35	0.16	-	-	1.02	0.33	2.36
Acetoin	-	-	nil	nil	nil	nil	-	-	nil	nil	-
Diacetyl	-	-	nil	nil	nil	nil	-	-	nil	nil	-
2,3-butanediol	-	-	nil	nil	nil	nil	-	-	Tr.	Tr.	-
pH	5.88	6.09	4.16	4.12	4.88	4.90	6.10	6.10	3.67	4.18	3.91

* After hydrolysis of WSC.

TABLE II

Percentage losses during Ensilage

	Experiment 1 A	Experiment 1 B	Experiment 1 C	Experiment 1 D	Experiment 2 A Total	Experiment 2 A Effluent	Experiment 2 B
(a) Total edible loss							
Dry matter	6.8	10.4	7.1	6.7	18.2	6.4	7.5
(b) Loss from the silo							
Dry matter	1.9	3.7	2.1	1.1	16.0	6.4	7.5
Total N	+ 0.5	+ 2.7	+ 2.7	3.5	15.6	9.3	6.4
Water-soluble carbohydrates	51.4	47.4	23.7	4.8	93.9	4.8	66.4

(a) Including weight of waste

(b) Excluding weight of waste

TABLE III

Recoveries of some constituents during ensilage in Experiment I

(weights in kg based on material below marker sheet)

	A			B			C			D		
	In	Out	Loss	In	Out	Loss	In	Out	Loss	In	Out	Loss
Dry matter	250.0	249.7	0.3	243.5	240.0	3.5	349.4	348.6	0.8	342.7	345.6	+2.9
*Glucose	15.3	6.2	9.0	14.8	5.8	9.0	18.4	15.7	2.7	18.0	18.3	+0.3
≠Fructose	39.8	15.7	24.1	39.0	15.4	23.6	51.8	31.7	20.1	50.8	40.1	10.7
Mannitol	-	19.7	-	-	17.8	-	-	13.6	-	-	11.1	-
Ethanol	-	1.1	-	-	0.8	-	-	1.2	-	-	0.6	-

*Including glucose present in sucrose

≠Including fructose present in sucrose and fructosans

TABLE IV

Percentage digestibility (D), percentage of digestible nutrients (DN) and energy values of true dry matter (Experiment 1)

[mean values of triplicate trials (grass) and duplicate trials (silage)]

	Grass				Silages							
	AB		CD		A		B		C		D	
	D	DN	D	DN	D	DN	D	DN	D	DN	D	DN
Organic matter	75.4	69.1	72.7	66.3	76.4	69.8	75.3	68.9	72.7	66.3	74.6	67.9
Crude protein	66.7	7.9	59.7	7.4	66.6	8.1	64.6	8.2	63.2	8.2	63.7	7.7
*ME (kcal/g)	-	2.50	-	2.40	-	2.59	-	2.54	-	2.52	-	2.50
Starch Equivalent	-	62.4	-	59.5	-	64.0	-	62.7	-	62.1	-	61.5

Intakes (g. dry matter/kg $W^{0.73}$)

	Grass		Silages			
	AB	CD	A	B	C	D
1.	86.1	58.1	76.4	74.7	77.0	71.4
2.	70.7	66.8	74.5	65.7	71.6	62.3
3.	75.7	66.6	-	-	-	-
mean	77.5	63.8	75.4	70.2	74.3	66.9

* Calculated from TDN.

TABLE V

Changes in carbohydrates and organic acids in laboratory silos

Experiment 1

	Fresh grass (20.3% d.m.)				Wilted grass (34.7% d.m.)				Wilted grass (48.5% d.m.)			
	Original material	30h	14 days	58 days	Original material	30h	14 days	58 days	Original material	30h	14 days	58 days
Carbohydrates												
Glucose	5.7	5.5	3.2	2.3	4.5	4.8	3.8	2.6	4.2	5.0	4.9	4.2
Fructose	4.4	4.7	2.6	0.5	4.2	5.1	6.3	5.8	4.2	6.4	7.2	8.3
Sucrose	6.7	2.1	Nil	Nil	3.2	1.7	Nil	Nil	2.0	tr.	Nil	Nil
*Fructosans	7.1	3.5	tr.	Nil	6.8	3.1	tr.	Nil	5.2	2.2	tr.	Nil
Xylose	Nil	Nil	tr.	tr.	Nil	Nil	tr.	0.6	Nil	Nil	tr.	0.5
Galactose	Nil	Nil	tr.	tr.	Nil	Nil	tr.	0.5	Nil	Nil	tr.	0.5
Acids												
Butyric	Nil	Nil	1.70	1.71	Nil	Nil	Nil	0.63	Nil	Nil	Nil	0.1
Propionic	Nil	tr.	0.73	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Acetic	Nil	0.71	0.83	1.17	Nil	0.85	2.02	1.35	Nil	tr.	0.32	1.17
Formic	Nil	Nil	0.10	Nil	Nil	Nil	tr.	0.09	Nil	Nil	Nil	Nil
Succinic	Nil	0.79	0.92	0.99	Nil	0.21	0.11	0.14	Nil	Nil	Nil	Nil
Lactic	Nil	4.15	11.16	13.86	Nil	3.50	7.80	10.90	Nil	0.30	1.73	3.72
Malic	1.17	Nil	Nil	Nil	1.15	Nil	Nil	Nil	1.18	0.30	Nil	Nil
Citric	0.53	Nil	Nil	Nil	0.53	Nil	Nil	Nil	0.51	tr.	Nil	Nil
pH	5.9	5.1	4.2	4.0	5.9	5.1	4.3	4.1	6.1	5.8	4.8	4.8

*Excluding oligosaccharides

TABLE VI

Changes in carbohydrates and organic acids in laboratory silos

(Experiment 2)

(all figures given as % dry matter (d.m.))

	Fresh grass (15.5% d.m.)					Wilted grass (30.5% d.m.)				
	Original material	24h	48h	7 days	36 days	Original material	24h	48h	7 days	36 days
Carbohydrates										
Glucose	7.0	5.2	3.9	1.1	1.1	6.3	5.7	5.7	3.5	2.9
Fructose	5.0	6.3	7.8	6.6	4.6	7.7	7.7	7.7	5.5	5.2
Sucrose	6.7	4.3	Nil	Nil	Nil	5.4	1.2	0.3	Nil	Nil
*Oligosaccharides	2.9	3.0	3.3	1.2	1.2	1.9	1.6	0.9	0.9	0.8
Fructosans	6.5	5.7	5.2	4.5	3.9	5.9	5.6	5.4	3.3	2.4
Mannitol	Nil	Nil	2.2	5.6	6.3	Nil	tr.	tr.	6.8	7.0
Xylose	Nil	Nil	Nil	tr.	tr.	Nil	Nil	Nil	Nil	0.3
Galactose	Nil	0.2	0.6	1.3	0.9	Nil	tr.	tr.	tr.	0.4
Acids										
Butyric	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Propionic	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Acetic	tr.	0.11	0.22	0.83	2.21	Nil	0.11	0.40	1.40	2.06
Formic	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Succinic	tr.	0.45	0.45	0.31	Nil	Nil	Nil	Nil	Nil	Nil
Lactic	Nil	tr.	1.69	8.44	11.62	Nil	tr.	1.00	2.78	6.91
Malic	1.75	0.80	Nil	Nil	Nil	1.71	1.16	1.01	Nil	Nil
Citric	0.65	tr.	Nil	Nil	Nil	0.77	0.50	0.48	Nil	Nil
Fumaric	0.19	Nil	Nil	Nil	Nil	0.09	Nil	Nil	Nil	Nil
Malonic	0.13	2.03	1.61	1.43	tr.	0.13	0.54	0.52	0.48	0.38
Glycollic	Nil	Nil	Nil	Nil	Nil	0.14	Nil	Nil	Nil	Nil
pH	6.10	6.40	5.12	4.11	4.06	6.10	6.60	6.40	4.91	4.37

*Excluding sucrose, but including short chain fructosans

TABLE VII

Fermentation pathways used in calculations

Ref.

1. 1 Glucose $\longrightarrow$ 2 Lactic acid
2. 1 Fructose $\longrightarrow$ 2 Lactic acid
3. 1 Glucose $\longrightarrow$ 1 Lactic acid + 1 Ethanol + 1 Carbon dioxide
4. 3 Fructose $\longrightarrow$ 1 Lactic acid + 2 Mannitol + 1 Acetic acid + 1 Carbon dioxide
5. 2 Fructose + 1 Glucose $\longrightarrow$ 1 Lactic acid + 2 Mannitol + 1 Acetic acid + 1 Carbon dioxide
6. 2 Citric acid $\longrightarrow$ 1 Lactic acid + 3 Acetic acid + 3 Carbon dioxide
7. 1 Malic acid $\longrightarrow$ 1 Lactic acid + 1 Carbon dioxide

TABLE VIII

Weights (kg) of sugars and fermentation products calculated from pathways in Table VII

(Weights based on dry matter values given in Table III)

Pathway	Glucose	Fructose	Lactic acid	Acetic acid	Ethanol	Mannitol	CO_2
Silo A							
1	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-
3	4.24	-	2.12	-	1.12	-	1.04
4	-	14.96	2.49	1.16	-	10.08	1.22
5	4.77	9.53	2.39	1.59	-	9.64	1.17
6	-	-	0.31	0.62	-	-	0.46
7	-	-	1.93	-	-	-	0.94
Total	9.01	24.49	9.24	3.87	1.12	19.72	4.83
Found	9.01	24.02	13.61	5.32	1.12	19.72	-
Silo B							
1	-	-	-	-	-	-	-
2	-	3.04	3.04	-	-	-	-
3	3.28	-	1.64	-	0.84	-	0.80
4	-	8.99	1.50	1.00	-	6.06	0.73
5	5.79	11.57	2.90	1.93	-	11.70	1.42
6	-	-	0.29	0.58	-	-	0.43
7	-	-	1.93	-	-	-	0.94
Total	9.07	23.60	11.30	3.51	0.84	17.76	4.32
Found	9.07	23.60	12.96	4.94	0.84	17.76	-
Silo C							
1	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-
3	4.77	-	2.39	-	1.22	-	1.17
4	-	20.17	3.36	2.24	-	13.59	1.64
5	-	-	-	-	-	-	-
6	-	-	0.42	0.83	-	-	0.61
7	-	-	2.77	-	-	-	1.35
Total	4.77	20.17	8.94	3.07	1.22	13.59	4.77
Found	2.66	20.07	5.96	4.01	1.22	13.59	-

TABLE VIII continued

Pathway	Glucose	Fructose	Lactic acid	Acetic acid	Ethanol	Mannitol	CO_2
Silo D							
1	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-
3	2.15	-	1.08	-	0.55	-	0.53
4	-	16.41	2.73	1.82	-	11.06	1.34
5	-	-	-	-	-	-	-
6	-	-	0.41	0.82	-	-	0.60
7	-	-	2.71	-	-	-	1.33
Total	2.15	16.41	6.93	2.64	0.55	11.06	3.80
Found	+0.33	10.70	3.21	2.35	0.55	11.06	-

SECTION B. MINERAL AND DIGESTIBILITY STUDIES

21.

Edinburgh and East of Scotland College of Agriculture, Miscellaneous Publication No. 159

APPLICATION OF MAGNESIUM SULPHATE TO GRASS FOR SILAGE AS A MEANS OF PREVENTING HYPOMAGNESAEMIC TETANY

P. McDonald and T. H. Jackson

Edinburgh and East of Scotland College of Agriculture

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APPLICATION OF MAGNESIUM SULPHATE TO GRASS FOR SILAGE AS A MEANS OF PREVENTING HYPOMAGNESAEMIC TETANY

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The incidence of hypomagnesaemic tetany in cattle and sheep can be reduced considerably by increasing the dietary intake of magnesium (Allcroft, R., 1954; Allcroft, W. M., 1947; Bartlett *et al.*, 1954; Stewart, 1954) and the commonest method of supplementing the ration is to provide a mineral mixture containing magnesium oxide. While the feeding of these mineral supplements to animals wintered indoors is relatively straightforward, ensuring that animals grazing on hill and marginal land will obtain a regular supply of the mineral raises a problem. Where animals are receiving silage in addition to natural grazings during the winter, the inclusion of a magnesium compound in the grass at the time of ensiling is one method of increasing the magnesium content of the diet. The oxide and carbonate of magnesium are probably the safest compounds for feeding to stock, but unfortunately these are likely to have an adverse effect on the preservation, resulting in inferior silage. Experiments carried out in small silo units at Boghall Farm proved this to be the case (*E.E.S.C.A. Ann. Rep.* 1955). Magnesium sulphate is likely to have little effect upon the preservation of silage, and although this compound is not ideally suitable as a magnesium supplement owing to its laxative properties, it was decided to investigate the value of using it in restricted quantity and intimately mixed with the silage, as a suitable means of preventing hypomagnesaemia when fed during the winter to a herd of 18 outwintered Galloway cows.

MATERIAL AND METHODS

Silage. The experiment was designed to study two methods of applying magnesium sulphate to grass for silage. One treatment consisted of spraying the herbage some 4 days prior to cutting, while the other incorporated the magnesium sulphate in the cut grass at the time of making the silage.

A 3-year-old grass and clover ley of 14 acres was set aside for silage on 14th May 1954. The field was given a dressing of 2 cwt./acre of a compound fertiliser (8% N; 8% P_2O_5; 12% K_2O) and 10 cwt./acre of 'Basic Slag' (18% P_2O_5). On 10th June, 4 days before cutting began, one third of the field was sprayed with a solution of kieserite ($MgSO_4.H_2O$) at the rate of 28 lb. of the salt dissolved in 10 gallons of water per acre. Cutting began on 14th June and finished on 19th June. The estimated yield per acre was 3 tons of fresh material.

A surface clamp silo was used for this experiment; it was divided into 3 sections which were designated A, B and C. Section A, the control, was filled with untreated grass. Section B was filled with grass sprayed at the silo with a solution of kieserite at the rate of 5 lb. of the salt dissolved in 4 gallons of water for each ton of fresh herbage. Section C was filled with the grass which had previously been sprayed with kieserite as described above. The clamp was sealed with lime and roofed with aluminium sheeting. The total quantity of herbage ensiled was estimated at 42 tons.

Herd Management. The Galloway herd of 18 animals, which were due to calve in April 1955, was wintered on Castlelaw Hill, 1,000 feet above sea-level. The natural herbage on the hill was mainly *Festuca spp*., some *Agrostis spp*. and *Nardus stricta*, with patches of heather on the high ground. The cattle were fed oat straw (8-10 lb/head) during December and were given daily access to the silage clamp from 4th January until 7th March, after which complementary grazing (2 hours on a pasture previously dressed with 5 cwt. 'Nitro-chalk'/acre and then back onto the hill) was carried out until the end of May. Hay replaced the oat straw during April; concentrates, consisting of 1 part linseed to 6 parts crushed oats, were fed at the rate of 3 lb./head per day after 7th March.

Samples of silage were taken for analysis by means of a corer during the experimental feeding period. Monthly blood samples were taken from individual animals with the exception of the month of April, when the animals were calving.

Blood samples of the 18 cows were analysed for magnesium in November and December before silage feeding began. The cows were then allocated to 3 groups of 6 each, with each group representing a similar mean and range of blood magnesium values. Each group was allocated to one of the silage sections.

RESULTS AND DISCUSSION

The composition of the grass and silages is given in the Table. The silages were not of good quality, as reflected by the pH values,

TABLE 1

Composition of grass and silages

		Dry matter (%)	pH	Crude protein in dry matter (%)	Mg in dry matter (%)
Grass	A Control	18·9	—	16·6	0·16
	B Sprayed in field	18·2	—	16·7	0·18
	C *Sprayed at silo	18·7	—	16·4	0·37
Silage	A Control	20·8	5·5	17·4	0·19
	B Sprayed in field	20·2	5·8	15·9	0·24
	C Sprayed at silo	18·7	5·6	17·9	0·32

* Calculated.

and all three were of high butyric acid content. A possible contributory cause of the inferior preservation, in addition to the relatively high protein content, was inefficient consolidation, owing to the division of the silo into 3 sections. The magnesium contents of the untreated grass (A) and the 'sprayed in the field' (C) grass did not differ widely (0·02%). The similarity in composition is probably due to the removal of some of the magnesium salt from the standing crop by rain which fell the day after the herbage had been sprayed.

The mean blood magnesium values for each group of cows are shown in the Figure.

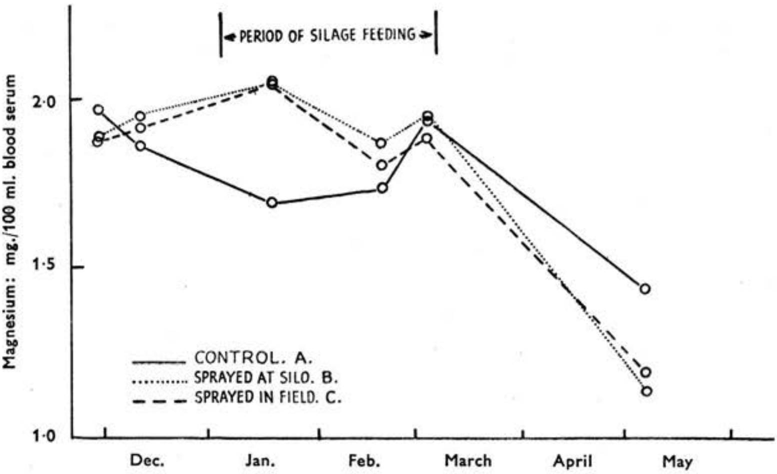

FIG. Variation in mean blood magnesium values of three groups of Galloway cows.

The spraying of a crop in the field with a solution of magnesium sulphate, as distinct from spraying cut grass for silage, is probably a safer method of increasing the magnesium content of the herbage because of absorption of the salt by the living plant tissues; unfortunately, however, the whole operation is at the mercy of the weather and large losses of magnesium sulphate are liable to occur if rain falls between the time of spraying and ensiling. It is evident from the magnesium values in the Table that such 'leaching' had occurred in this experiment. Further losses of the salt are liable to occur via the effluent, the extent of these depending upon the amount of moisture in the original crop and on the degree of consolidation at the time of ensiling. The dry matter content of the control grass at the time of cutting was only 18·9% and because of this a considerable amount of effluent had drained from the silo before the clamp was opened. The relatively low magnesium content of the silage made from herbage sprayed at the pit is an indication that losses of the salt had occurred in this way. Partial wilting of the crop prior to ensiling would be one method of reducing effluent losses of the magnesium sulphate.

In addition to the variation of blood magnesium values which occurs in individual animals within a herd, it is also common to find a seasonal variation which, according to Stewart (1954), may be of the order of from 3·0 mg. to 1·5 mg. per 100 ml. of serum; the actual symptoms of tetany are liable to develop when the blood magnesium falls from 1·5 mg. to less than 1·0 mg. Stewart has stated that 'any animal which has a low blood magnesium of the 1·5 mg. level is a potential case of tetany and may become a case proper if exposed to any undue excitement'. Three animals (one in each group) showed low blood magnesium values throughout the whole period of the trial and would, at the beginning of the experiment, come under the category of 'potential tetany cases' according to the above standards.

Several other animals showed blood magnesium values low enough in May to be described as hypomagnesaemic. No clinical cases of tetany occurred.

It can be seen from the Figure that the control group on 17th January, some 13 days after the beginning of the silage feeding, showed magnesium values which were considerably lower than those in the other two groups; this difference is statistically significant at the 5% level. Although the mean magnesium values of the control group in February was lower than those of the other two groups, the difference here is not significant. The final blood samples taken while the animals were on the silage diet in March showed little difference in mean serum values; at this stage, however, the animals were grazing the bottom of the clamp and it is likely that some diffusion of magnesium sulphate had occurred from the centre section into the control.

The blood samples taken in May, after the animals had calved, are interesting in that although all animals showed low magnesium values, those of the control group were the highest, with a mean value of 1·44 compared with 1·13 and 1·19 for groups B and C respectively. However, the range of magnesium values between individuals within groups is quite considerable, and although the mean values differ the difference is not statistically significant.

The results indicate that the pre-feeding of magnesium-sulphate-treated silage did not enable the animal to build up body reserves in order to keep the blood magnesium values near the normal level during the critical period. This illustrates the danger in feeding magnesium supplements to stock and discontinuing before or during the critical periods of the season.

In spite of the fact that the silage was of the butyric acid type the animals appeared to find the products palatable and no differences in palatability were observed between groups. No sign of scouring was seen in the groups of animals on the magnesium-sulphate-treated silages, although this might not have been the case had effluent losses not occurred or had distribution of the salt been less even throughout the herbage.

While it is impossible to calculate accurately the intake of silage by individual animals, because of the self-feed system, estimated figures put this value at about 70 lb. per head per day.

One interesting fact which can be seen from the results is that the magnesium content of the control silage (0·19%) was higher than that of the original grass (0·16%), i.e. the process of ensiling had tended, owing to fermentation losses, to produce a material slightly richer in natural magnesium.

SUMMARY

Two methods of applying magnesium sulphate to grass intended for silage have been described. The application of the salt in solution to grass prior to cutting was not highly successful owing to inclement weather conditions. The other method adopted, viz. applying the salt by spraying the cut herbage at the time of ensiling, proved to be more successful in producing a silage of higher magnesium content although losses of magnesium sulphate had occurred via the effluent. Nevertheless both groups of animals consuming the magnesium-

treated silages showed significantly higher blood magnesium values than the control group during the first month of feeding.

The blood magnesium values dropped in all groups after the silage was finished and the animals were grazing young grass in the spring, indicating that the pre-feeding of magnesium-sulphate-treated silages did not enable the animal to build up body reserves in order to keep the blood magnesium values near the normal level during the critical spring period.

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EFFECTS OF NITROGEN AND POTASSIUM FERTILISERS ON THE MINERAL STATUS OF PERENNIAL RYEGRASS (*LOLIUM PERENNE*). I.—Mineral Content

By H. RAHMAN, P. McDONALD and K. SIMPSON

Results are given for the effects of ammonium nitrate applied at two levels and potassium sulphate at one level on the nitrogen, calcium, magnesium, sodium, potassium, phosphorus, sulphur and chlorine content of perennial ryegrass grown pure and in the presence of clover. Three cuts were taken over two seasons and statistical analyses of the results are presented for each cut.

Application of nitrogen fertilisers increased the N content of the ryegrass (chiefly the non-protein nitrogen). Sodium contents were increased at all cuts and potassium in the second and third cuts, after application of nitrogen. Chlorine content was decreased and phosphorus increased (at second and third cuts). Variable magnesium contents were obtained, possibly due to rainfall conditions.

Introduction

A number of workers, including Bartlett *et al.*[1] and Brouwer,[2] have commented on the possible adverse effect of heavy dressings of nitrogenous and potassic fertilisers on grassland. These workers have stressed the probability of increasing the incidence of hypomagnesaemic tetany in ruminant animals grazing pastures overdosed with these fertilisers. Although a number of workers including Stewart & Holmes,[3] Bosch[4] and Thomas & Thompson[5] have provided valuable data on the effects of different manurial treatments on the mineral composition of grasses, there is still a considerable lack of information on the effects of fertiliser treatment with specific reference to magnesium.

It is clear that the mineral composition of a sward is dependent on many factors other than the mineral status of the soil and such factors as botanical make-up, stage of growth, and season cannot be overlooked. In this investigation the effect on grass of two levels of nitrogenous fertiliser and one of potassium has been studied in herbage sampled at three different times of the year from replicated plots. Attention has been given to a single species, namely perennial ryegrass, although the effect of the presence of clover on the ryegrass has also been studied.

Experimental

The experimental site was at Bush Estate, Midlothian. The soil of the experimental area is a sandy clay loam, derived from water-worked till with drainage impeded at 2 ft. to 2 ft. 6 in. At the time of the experiment the average soil pH was 6·0. The available phosphorus and potassium contents were low.

The design was a $3 \times 2 \times 2$ factorial with three replications, three rates of nitrogen (N_0, N_1 and N_2) being applied with and without potassium (K_0 and K). The species of grass used was S.100 perennial ryegrass. One half of the plots was sown pure (C_0) and the other half with S.24 white clover (C). The treatments were randomised in six sub-blocks so that the interaction of KC and the triple interaction NKC were partially confounded with blocks. These interactions were almost always non-significant and for the purpose of the statistics presented below the degrees of freedom allocated to them have been included with those for error.

All nitrogen treatments were applied in the form of ammonium nitrate and the potassium was applied as potassium sulphate. Table I shows the time and rate of application of fertilisers.

Table I

Fertiliser treatments in lb. of N and K/acre

Treatment	20/4/56	20/7/56	23/12/56	7/3/57
K (lb. K/acre)	188	—	188	—
N_1 (lb. N/acre)	84	84	—	84
N_2 (lb. N/acre)	168	168	—	168

The grass and clover were sown on 15/5/56 at the rates of 30 lb. and 2 lb. per acre, respectively. Two cuts were taken during 1956 on 18/7/56 and 20/8/56 and one during the following year on 23/5/57. In these experiments only grass was analysed and the samples were taken for analysis after separation from clover and weeds. Weed competition was severe in the early weeks and it was necessary to hand-weed all plots before the first cut was taken. Soil contamination was removed by rapid washing with water and the samples were placed on wire netting to drain prior to drying in an electric oven for 24 h. at 100°.

Methods of analysis

Calcium and magnesium were determined by the methods of analysis for stock feeds laid down by the A.O.A.C.;[6] phosphorus by the method of Richards & Godden;[7] chlorine by Caldwell & Moyer's modification[8] of Husband & Godden's method; sulphur by the procedure of Marston[9] and sodium and potassium by the flame photometer method of Peach & Tracey.[10]

Results

Nitrogen content

The effect of treatments on the percentage of total and non-protein nitrogen is shown in Table II. In all the tables, the main effects of nitrogen, potassium and clover treatments are shown in the form of two-way tables giving also the interactions of nitrogen with both potassium and clover. L.S.D. (least significant differences) ($P = 0{\cdot}05$) are given below the main table. If the differences were non-significant, no L.S.D. is given. It should be noted that the nitrogen treatments greatly affected the amount of clover in the sward, the N_0 plots having a high proportion of clover, N_1 a moderate amount and N_2 plots very little. NC interactions may, therefore, often be significant on account of the lack of clover on high-nitrogen-treated plots.

Table II

Effect of treatments on the percentage of total and non-protein nitrogen in the dry matter of ryegrass

Total nitrogen in dry matter, %

Treatment	1st cut N_0	N_1	N_2		2nd cut N_0	N_1	N_2		3rd cut N_0	N_1	N_2	
K_0	1·65	1·68	2·20	1·84	2·53	2·93	3·27	2·91	1·80	2·48	3·08	2·46
K	1·77	1·85	2·08	1·90	2·27	2·80	3·08	2·71	1·75	2·37	2·98	2·37
C_0	1·48	1·75	2·27	1·83	2·02	2·83	3·17	2·67	1·70	2·50	2·97	2·39
C	1·93	1·78	2·02	1·91	2·78	2·92	3·19	2·96	1·85	2·35	3·10	2·43
	1·71	1·77	2·14		2·40	2·87	3·18		1·78	2·43	3·03	
L.S.D.	Main N = 0·086 Main C = 0·071 Interaction NK, NC = 0·123				Main N = 0·121 Main K, C = 0·099 Interaction NC = 0·172				Main N = 0·103 Main K = 0·084 Interaction NC = 0·147			

Non-protein nitrogen in dry matter, %

Treatment	1st cut N_0	N_1	N_2		2nd cut N_0	N_1	N_2		3rd cut N_0	N_1	N_2	
K_0	0·29	0·38	0·56	0·41	1·00	1·54	1·58	1·38	0·53	0·72	0·92	0·72
K	0·25	0·47	0·55	0·42	0·72	1·42	1·32	1·15	0·49	0·65	0·78	0·64
C_0	0·20	0·35	0·55	0·36	0·50	1·46	1·45	1·14	0·49	0·75	0·85	0·70
C	0·34	0·51	0·56	0·47	1·22	1·50	1·47	1·39	0·53	0·61	0·85	0·67
	0·27	0·43	0·55		0·86	1·48	1·46		0·51	0·68	0·85	
L.S.D.	Main N = 0·107 Main C = 0·088				Main N = 0·112 Main K, C = 0·091 Interaction NC = 0·158				Main N = 0·090 Main K = 0·074			

The expected increase in the total nitrogen with increased nitrogen applications was found in all three cuts, although only the higher dressing gave a significant increase at the first cut. In both 1956 cuts, practically the whole of the increase in total nitrogen was accounted for by the highly significant increases in non-protein nitrogen brought about by application of nitrogen. In 1957, however, only about one-third of the extra uptake was in the form of non-protein nitrogen and considerable increases in true protein resulted.

Except at the first cut where it had no overall effect, the potassium treatment depressed both total and non-protein nitrogen. The reduction in non-protein nitrogen more than accounted for that in total nitrogen at both the second and third cuts. In the first cut there was an interaction between nitrogen and potassium treatments on the total nitrogen percentage, potassium treatments stimulating nitrogen uptake at the N_0 and N_1 levels and depressing it at the N_2 level.

The presence of clover along with the ryegrass increased the percentage of total nitrogen at all cuts (3rd cut not significant). This increase was also associated with one in non-protein nitrogen. The nitrogen–clover interaction was significant at all three cuts, the increase in nitrogen content of the ryegrass being associated with the presence of clover, while on the nitrogen-treated plots where clover was lacking it was non-significant. At the second cut, the increased nitrogen content of the grass, associated with the presence of clover, could be accounted for by the increase in non-protein nitrogen, but at the other two cuts the non-protein nitrogen increased by only a small fraction of the total nitrogen increase.

Table III shows the effect of treatments on the percentage of the cations magnesium, calcium, sodium and potassium in the dry matter of ryegrass at all three cuts.

Magnesium content

The effect of nitrogen applications on the magnesium content of the ryegrass altered as the sward became established. In the first cut, where weed competition was severe, the overall effect was a reduction in the percentage of magnesium particularly at the N_1 level. This picture was complicated by NK and NC interactions. The increase in magnesium content of ryegrass by nitrogen application occurred only in the presence of potassium, a depression being recorded when nitrogen only was used. Also, nitrogen applied to the pure ryegrass, and the presence of clover, depressed the magnesium content.

At the second cut, nitrogen treatments had little effect on the magnesium content but a small significant increase was brought about by the introduction of clover. At the third cut, however, on the well-established sward in 1957, increasing nitrogen treatments had a striking effect in increasing the magnesium content. For the N_1 and N_2 treatments the increase over control was about 16 and 33% and was independent of the presence of potassium fertilisers or clover.

The effect of potassium fertilisers in reducing the magnesium content of grass was found only at the first cut and then only in the absence of or at the first level of application of nitrogen.

Calcium content

Nitrogen treatments decreased the percentage of calcium in the ryegrass at the first cut. This effect was also observed at the second cut but only in the clover–ryegrass plots. There was a strong nitrogen–clover interaction at this cut and on pure ryegrass plots the nitrogen treatments strikingly increased the calcium content of the herbage. At the third cut (May 1957) nitrogen treatments increased the calcium content irrespective of the presence of clover.

Potassium treatments steadily and considerably depressed the percentage of calcium in the ryegrass. The overall effects of clover were not significant at any cut.

Sodium content

The effect of clover on the sodium content of the ryegrass was not significant at any stage, and there was no interaction of nitrogen with clover. Nitrogen and potassium treatments, however, both had very marked effects and interacted strongly.

The potassium treatments progressively depressed the sodium content from the first to the third cut, the depression being 13, 38 and 60% for the three cuts. It did not occur at any cut unless nitrogen had also been applied but was most marked at either the first or second level of nitrogen application. The sodium content of the N_2K_0 ryegrass at the third cut was in fact more than three times that from the N_2K_1 plots.

Nitrogen applications, on the other hand, strongly increased the percentage of sodium in the ryegrass at all three cuts. Again, this was not an independent effect, being stronger at all cuts in the absence of potassium treatment.

Table III

Effect of treatments on the percentage of Mg, Ca, Na and K in the dry matter of ryegrass

MAGNESIUM

Treatment	1st cut N_0	1st cut N_1	1st cut N_2		2nd cut N_0	2nd cut N_1	2nd cut N_2		3rd cut N_0	3rd cut N_1	3rd cut N_2	
K_0	0·208	0·175	0·178	0·187	0·217	0·215	0·225	0·219	0·122	0·147	0·167	0·145
K	0·153	0·163	0·170	0·162	0·208	0·217	0·212	0·212	0·122	0·143	0·162	0·142
C_0	0·192	0·165	0·175	0·177	0·200	0·200	0·217	0·206	0·123	0·148	0·162	0·144
C	0·170	0·173	0·173	0·172	0·225	0·232	0·220	0·226	0·120	0·142	0·167	0·143
	0·181	0·169	0·174		0·213	0·216	0·218		0·122	0·145	0·164	
L.S.D.	Main N = 0·0085 Main K = 0·0085 Interaction NK, NC = 0·0120				Main C = 0·0075 Interaction NC = 0·013				Main N = 0·0078			

CALCIUM

Treatment	1st cut N_0	1st cut N_1	1st cut N_2		2nd cut N_0	2nd cut N_1	2nd cut N_2		3rd cut N_0	3rd cut N_1	3rd cut N_2	
K_0	0·553	0·518	0·497	0·523	0·530	0·538	0·527	0·532	0·447	0·498	0·527	0·491
K	0·483	0·465	0·452	0·467	0·475	0·472	0·490	0·479	0·408	0·397	0·430	0·412
C_0	0·523	0·493	0·467	0·494	0·463	0·500	0·538	0·501	0·420	0·447	0·477	0·448
C	0·513	0·490	0·482	0·495	0·542	0·510	0·478	0·510	0·435	0·448	0·480	0·454
	0·518	0·492	0·474		0·503	0·505	0·508		0·428	0·448	0·478	
L.S.D.	Main N = 0·023 Main K = 0·019				Main K = 0·152 Interaction NC = 0·026				Main N = 0·017 Main K = 0·014 Interaction NK = 0·024			

SODIUM

Treatment	1st cut N_0	1st cut N_1	1st cut N_2		2nd cut N_0	2nd cut N_1	2nd cut N_2		3rd cut N_0	3rd cut N_1	3rd cut N_2	
K_0	0·083	0·128	0·218	0·143	0·060	0·135	0·207	0·134	0·052	0·130	0·227	0·139
K	0·105	0·105	0·168	0·126	0·050	0·063	0·138	0·084	0·053	0·052	0·065	0·057
C_0	0·080	0·128	0·167	0·131	0·052	0·102	0·170	0·108	0·053	0·110	0·130	0·101
C	0·108	0·105	0·203	0·139	0·058	0·097	0·175	0·110	0·052	0·082	0·152	0·095
	0·094	0·117	0·193		0·055	0·099	0·173		0·053	0·096	0·146	
L.S.D.	Main N = 0·026 Interaction NK = 0·036				Main N = 0·012 Main K = 0·010 Interaction NK = 0·017				Main N = 0·016 Main K = 0·013 Interaction NK = 0·023			

POTASSIUM

Treatment	1st cut N_0	1st cut N_1	1st cut N_2		2nd cut N_0	2nd cut N_1	2nd cut N_2		3rd cut N_0	3rd cut N_1	3rd cut N_2	
K_0	3·36	2·84	3·12	3·11	3·78	4·60	4·30	4·23	2·40	2·57	2·75	2·57
K	3·23	3·11	3·63	3·32	3·67	4·38	5·15	4·40	2·42	3·00	3·55	2·99
C_0	3·01	2·85	3·50	3·12	3·43	4·40	4·70	4·18	2·35	2·70	3·00	2·68
C	3·57	3·10	3·25	3·31	4·02	4·58	4·75	4·45	2·47	2·87	3·30	2·88
	3·29	2·98	3·37		3·73	4·49	4·73		2·41	2·78	3·15	
L.S.D.	Main N = 0·23 Main K = 0·19 Interaction NK, NC = 0·33				Main N = 0·27 Main C = 0·22 Interaction NC = 0·38				Main N = 0·11 Main K, C = 0·12 Interaction NK = 0·21			

Potassium content

At the two later cuts applications of nitrogen at both levels increased the content of potassium. The NK interaction was marked at all three cuts, the higher nitrogen treatment giving a better effect in all cases in the presence of potassium.

The application of potassium did not increase the potassium content of the ryegrass as much as was expected.

As stated above the nitrogen and potassium effects were interdependent and at all three cuts the potassium treatment increased the K content of the ryegrass most efficiently in the presence of the higher level of nitrogen.

The presence of clover had also a stimulating effect on the potassium content. This overall effect just failed to reach significance at the first cut, where it was strongest in the absence of nitrogen, but was significant at both the second and third cuts.

Table IV shows the effect of treatments on the percentage of phosphorus, sulphur and chlorine in the dry matter of ryegrass.

Table IV

Effect of treatments on the percentage of P, S and Cl in the dry matter of ryegrass

Treat-ment	1st cut				2nd cut				3rd cut			
	N_0	N_1	N_2		N_0	N_1	N_2		N_0	N_1	N_2	
PHOSPHORUS												
K_0	0·348	0·332	0·345	0·342	0·467	0·482	0·498	0·482	0·342	0·378	0·413	0·378
K	0·392	0·347	0·353	0·365	0·398	0·440	0·495	0·444	0·318	0·378	0·398	0·365
C_0	0·407.	0·338	0·338	0·361	0·412	0·430	0·483	0·442	0·330	0·373	0·402	0·368
C	0·333	0·340	0·360	0·344	0·453	0·492	0·510	0·485	0·330	0·383	0·410	0·374
	0·370	0·339	0·349		0·433	0·461	0·497		0·330	0·378	0·406	
L.S.D.	Main K, C = 0·016 Interaction NK, NC = 0·027				Main N = 0·024 Main K, C = 0·019 Interaction NK = 0·033				Main N = 0·019			
SULPHUR												
K_0	0·390	0·275	0·355	0·340	0·477	0·423	0·390	0·430	0·273	0·307	0·333	0·304
K	0·293	0·312	0·322	0·309	0·425	0·393	0·423	0·414	0·307	0·313	0·337	0·319
C_0	0·355	0·312	0·355	0·341	0·465	0·387	0·407	0·419	0·308	0·310	0·328	0·316
C	0·328	0·275	0·322	0·308	0·437	0·430	0·407	0·424	0·272	0·310	0·342	0·308
	0·342	0·293	0·338		0·451	0·408	0·407		0·290	0·310	0·337	
L.S.D.	Main N = 0·029 Main K, C = 0·024 Interaction NK = 0·041				Main N = 0·024 Interaction NK, NC = 0·034				Main N = 0·013 Main K = 0·011 Interaction NC = 0·019			
CHLORINE												
K_0	0·468	0·407	0·373	0·404	0·468	0·428	0·383	0·427	0·563	0·498	0·505	0·522
K	0·588	0·393	0·508	0·508	0·597	0·498	0·377	0·491	0·632	0·568	0·502	0·567
C_0	0·590	0·407	0·417	0·471	0·518	0·460	0·337	0·438	0·618	0·522	0·517	0·552
C	0·467	0·393	0·465	0·442	0·547	0·467	0·423	0·479	0·575	0·545	0·492	0·537
	0·528	0·400	0·441		0·533	0·463	0·380		0·597	0·533	0·503	
L.S.D.	Main N = 0·057 Main K = 0·047 Interaction NC = 0·081				Main N = 0·026 Main K, C = 0·022 Interaction NK, NC = 0·037				Main N = 0·031 Main K = 0·026 Interaction NK = 0·045			

Phosphorus content

At the first cut the overall effect of nitrogen treatments on the phosphorus content was not significant but, both in the presence of potassium and in the absence of clover, nitrogen treatments depressed the amount of phosphorus in the plant.

After the sward was well established, the two applications of nitrogen steadily increased the percentage of phosphorus in the dry matter. Apart from a slight NK interaction at the second cut, this effect was independent of either potassium applications or the presence of clover.

The potassium treatment increased the phosphorus content at the first sampling particularly where no nitrogen was applied. In the second cut this effect was strikingly reversed and a depression in the percentage of phosphorus was induced by the potassium treatment, the effect being most marked in the absence of nitrogen. A similar trend was noted at the third cut but the differences were not significant.

The effect of the presence of clover in the sward on the uptake of phosphorus by the ryegrass also changed as the sward became established. A very marked depression in P content, at the first cut, was induced by the presence of clover, probably because of the vigorous competition for phosphorus particularly where the clover was strongest (N_0 plots). At the second cut, however, the presence of clover strongly increased the phosphorus content of the ryegrass. The effect at the third cut, although slightly positive, was non-significant.

Sulphur content

The sulphur content of ryegrass was depressed by the lower level of nitrogen at the first sampling and by both levels at the later sampling in 1956. In both cases the effect was dependent on whether potassium sulphate had been applied or not and did not follow a regular pattern. At the third cut the sulphur content was increased by both nitrogen treatments and, as would be expected, by the potassium sulphate dressing.

Although the presence of clover had an overall effect only at the first cut, it depressed the sulphur content of ryegrass at all three cuts in the absence of nitrogen.

Chlorine content

The effect of treatments on chlorine content was rather unexpected. Nitrogen applications at all cuts produced a continuous and most marked reduction in chlorine content. At the second and third cuts the presence of potassium enhanced this depression. The overall effect of potassium treatments was to increase the chlorine content at all cuts. This effect became less marked as the sward became established, being 25%, 16% and 10% at the three cuts. As stated above the effects of potassium and nitrogen treatments were interdependent at the later cuts, the potassium salt having a very strong positive effect at the N_0 level and no effect at all at the N_2 level.

The effect of clover on chlorine uptake followed a similar pattern to that of the other 'anion elements'—depressing the percentage at the first cut, particularly where no N was applied, stimulating it at the second cut and being ineffective at the third cut.

Discussion

Bartlett *et al.*[1] have demonstrated the effect of heavy dressings of ammonium sulphate in increasing the incidence of hypomagnesaemic tetany in cows, although the condition is not an inevitable outcome of the use of this fertiliser. The importance of nitrogen metabolism in this condition has been mentioned by a number of workers including Derivaux[11] and Head & Rook.[12] In the present experiments nitrogen applications markedly increased the percentage of total nitrogen in the ryegrass at all cuts, but in the 1956 cuts the increase was accounted for almost entirely by increases in non-protein nitrogen (N.P.N.). The increase in N.P.N. associated with application of nitrogenous fertilisers may be a significant factor in the aetiology of hypomagnesaemic tetany.

The increase in total N content was always much more marked in the pure ryegrass plots. The N content of the ryegrass grown with clover on the N_0 plots was high. This effect was not shown in the N_1 and N_2 plots because of the suppression of clover by nitrogen treatments. A considerable amount of the increase in nitrogen associated with the presence of clover was in the form of N.P.N., especially in the second cut.

The effect of nitrogen applications in increasing the sodium content was highly significant at all cuts; this was more noticeable where no potassium had been applied. In the case of the potassium content of the grass, nitrogen applications increased the amount present only at the second and third cuts.

Since the magnesium content of herbage is regarded as being of considerable importance in the incidence of hypomagnesaemia it is necessary to consider the effects of treatments on this element in detail. The effect of high dressings of potassium fertilisers on the magnesium content of herbage has been widely investigated. Bartlett *et al.*[1] found that the application of potassium sulphate to grass tended to depress the magnesium content, although the effect on the grass was less marked when clover was present. A similar effect was observed by Stewart & Holmes[3] and by Brouwer[2] who found that the calcium content was also depressed.

Such a depression of magnesium by potassium fertilisers occurred only in the first cut in the present experiments and although it was highly significant in the N_0 plots, the significance was absent on the N_2 plots. Apart from this exception these results do not confirm those quoted above; it is difficult to explain this.

The mean percentage values for magnesium at the three cuts were 0·17, 0·22 and 0·14. These values vary considerably and suggest that weather conditions are having some effect. The rainfall at the site for May, June, July and August, 1956, was 1·21, 2·56, 5·12 and 8·04 in. respectively. More than half of the July rainfall fell in the last three days of the month and would not affect the first cut (18/7/56). In 1957 the monthly rainfall for March, April and May was 2·24, 1·17 and 1·68 in. It is obvious from this that the two low magnesium values were associated with low rainfall conditions. The higher value was obtained for grass grown during a very wet period.

The other obvious weather factor is temperature, but it is difficult to assess the effect of this except under carefully controlled conditions since Dijkshoorn & t'Hart[13a] have found that changes of temperature over a short period of time can affect the cationic composition of ryegrass.

The depression of calcium by potassium mentioned by Brouwer[2] was very marked at all cuts. This is not surprising considering the antagonism between these two elements in the soil.

The effect of nitrogen treatments in depressing the chlorine content was most striking. Dijkshoorn[13b] has commented that nitrates will depress the absorption of chlorine, sulphur and phosphorus. In the above experiments there is no consistent depression of sulphur and at the second and third cuts the phosphorus content is significantly increased. This finding is in agreement with results obtained by Simpson[14] for other crops. Reference has been made to the possible effect of rainfall variations on the magnesium content of grass. A similar relationship exists between phosphorus content and rainfall, the phosphorus figures for the second cut being much higher than for the other two. This agrees with work carried out by Simpson[15] on grass and other crops.

Conclusions

(1) Applications of ammonium nitrate increased the percentage of nitrogen in ryegrass at all cuts but this increase was mainly accounted for by non-protein nitrogen.

(2) Ryegrass grown with clover in the absence of nitrogenous fertilisers contained more nitrogen than when grown as a pure sward.

(3) The ammonium nitrate increased the sodium content at all cuts and generally increased phosphorus and potassium, but magnesium only at the third cut. This fertiliser decreased the percentage of chlorine in the ryegrass.

(4) Applications of potassium sulphate generally increased the potassium and chlorine contents and decreased calcium and sodium at all cuts. This fertiliser also generally decreased non-protein nitrogen, but only decreased magnesium at the first cut. The presence of clover generally increased the potassium and decreased the sulphur content of the ryegrass.

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EFFECTS OF NITROGEN AND POTASSIUM FERTILISERS ON THE MINERAL STATUS OF PERENNIAL RYEGRASS (*LOLIUM PERENNE*). II.*—Anion–Cation Relationships

By H. RAHMAN, P. McDONALD and K. SIMPSON

A number of anion–cation relationships including AA, TA, VA, EA (defined in the text), K/(Ca + Mg) and (K + Ca)/Mg were calculated from the results of a previous paper.[7] In addition a number of samples obtained from 'normal' and 'tetany' areas were examined. The results indicated that AA, TA and VA values and the K/(Ca + Mg) ratio were increased by both nitrogen and potassium fertiliser treatments. In a number of samples from 'tetany' areas the K/(Ca + Mg) ratios did not approach 2·2, although the AA values were generally high.

Introduction

Brouwer and co-workers[1] have discussed the importance of considering the acid–base equilibrium in herbage in relation to certain metabolic disturbances which occur in ruminant animals. Among the relationships considered (expressed in mequiv./kg. of dry matter) the following have been presented:

Alkali alkalinity (AA) = Na + K − Cl − S
Alkaline earth alkalinity (EA) = Ca + Mg − P
Total alkalinity (TA) = (Na + K − Cl − S) + (Ca + Mg − P)
Base excess (VA) = (Na + K − Cl − S) − (Ca + Mg − P)

In addition to the above the ratio K/(Ca + Mg) has been calculated. This ratio was employed by Verdeyen[2] who observed that grass tetany in dairy cattle was particularly liable to occur when the ratio in the pasture rose above 2·20. Kemp & t'Hart[3] have also confirmed this. Although the above ratio has been used by a number of workers, it seems logical to consider the antagonism of potassium and calcium to magnesium. With this in view the ratio (K + Ca)/Mg has also been considered.

Brouwer[1a] has studied the anion–cation relationships for a number of grass and hay samples and from the values obtained he has suggested the following tentative limits for normal grass and hay:

AA 100–375; EA 50–325; TA 300–550; VA − 150 to +250

The grasses obtained from tetany-inducing pastures were characterised by negative EA values and extremely high AA and VA values, the TA values being intermediate ones. Brouwer & van de Vliert[1b] have shown in one series of investigations that the VA value from tetany pastures may be very high and quoted values up to +600 mequiv./kg. of dry matter. It is interesting to compare this value with those from haemoglobinurea pastures inducing acid urine reaction in which the VA was negative (to −600). Brouwer[1a] has also stated that potassium fertilisers were mainly responsible for the fluctuation of the AA and EA values. Bosch[4] has stated that 'excess of a base' may be lowered by some fertilisers, so that diseases (e.g., scouring) are more likely to occur.

More recently Brandsma[5] has studied the mineral composition of grass on 14 'normal', healthy dairy farms. From a total of 150 samples he calculated the following:

AA 145 ± 109; EA 118 ± 80; TA 363 ± 128; VA 128 ± 142

The mean K, Ca and Mg values in the herbage dry matter from these farms were 2·97, 0·69 and 0·22%, respectively. The K/(Ca + Mg) ratio (in mequiv./kg. of dry matter) calculated from these figures is 1·44.

All these anion–cation relationships have been calculated from data collected from investigations in the Netherlands. There is little indication as to how these 'normal' values can be applied to farming conditions in Scotland, since as far as the authors are aware no similar

* Part I: *J. Sci. Fd Agric.*, 1960, **11**, 422

surveys have yet been completed. The main purpose of this paper is to consider some anion–cation relationships in perennial ryegrass grown under different nitrogen and potassium fertiliser treatments. The percentage composition of these ryegrass samples has been reported in a previous paper.[6] Throughout these calculations, one g.-atom of P has been valued at 3 in agreement with Brouwer's suggestion.[1a]

Experimental

The design of the experiment, sampling and analytical techniques have been described in Part I[6] and the composition of the perennial ryegrass samples has been calculated in mequiv./kg. of dry matter from the data there reported. In addition to the above, a number of samples were collected from different areas in S.E. Scotland. Nine samples of herbage were taken from apparently 'normal' farms in which no previous history of hypomagnesaemic tetany had been recorded. Eight samples of herbage were taken from five different farms on which deaths from tetany had occurred, while animals had been grazing. The samples were collected within a few days of the onset of tetany symptoms.

Results

The results are shown in Table I and the anion–cation relationships calculated from these results in Table II.

Table I

Composition of perennial ryegrass, mequiv./kg. of dry matter

Treatment*	1st cut (18.7.56)							2nd cut (20.8.56)							3rd cut (23.5.57)						
	Ca	Mg	Na	K	Cl	P	S	Ca	Mg	Na	K	Cl	P	S	Ca	Mg	Na	K	Cl	P	S
O	275	192	35	860	116	387	269	235	167	22	873	130	436	325	210	100	22	581	161	329	194
K	250	125	35	768	217	397	175	230	167	22	888	164	458	256	210	100	26	609	189	310	194
C	280	158	35	860	147	290	219	295	192	30	1075	135	465	269	235	100	26	632	158	339	150
KC	235	125	57	968	116	358	194	245	183	22	993	175	416	275	200	100	22	627	166	300	188
N_1	261	142	57	696	104	290	181	275	175	61	1239	107	416	244	260	125	74	625	138	368	194
N_1C	255	150	57	765	104	348	163	265	183	57	1118	135	513	288	240	117	48	686	141	368	194
N_1K	230	133	57	765	124	358	213	225	158	30	1021	152	416	238	190	117	22	765	155	358	194
N_1KC	235	142	35	827	116	310	181	250	200	26	1224	127	436	250	210	117	22	778	166	378	194
N_2	235	142	74	829	99	319	256	270	183	83	1129	96	494	250	260	133	91	653	152	397	206
N_2C	260	150	117	765	113	348	188	255	192	96	1070	121	474	238	270	142	104	748	133	407	213
N_2K	230	150	87	963	135	339	188	270	175	65	1265	104	445	256	220	133	30	876	138	387	206
N_2KC	220	133	61	896	149	348	213	220	175	57	1354	127	513	269	210	133	26	934	144	387	219

Table II

Cation–anion relationships in perennial ryegrass calculated from mequiv./kg. of dry matter

Treatment*	1st cut						2nd cut						3rd cut					
	AA	EA	TA	VA	K/(Ca+Mg)	(K+Ca)/Mg	AA	EA	TA	VA	K/(Ca+Mg)	(K+Ca)/Mg	AA	EA	TA	VA	K/(Ca+Mg)	(K+Ca)/Mg
O	510	80	590	430	1·84	5·91	440	−34	406	474	2·17	6·63	248	−19	229	267	1·87	7·91
K	411	−22	389	433	2·05	8·14	490	−61	429	551	2·24	6·69	252	0	252	252	1·96	8·19
C	530	148	678	382	1·96	7·22	566	22	588	544	2·21	7·14	350	4	354	346	1·89	8·67
KC	715	2	717	713	2·69	9·62	565	12	577	553	2·32	6·77	295	0	295	295	2·09	8·27
N_1	468	113	581	355	1·73	6·74	949	34	983	915	2·75	8·65	367	17	384	350	1·62	7·08
N_1C	455	57	512	398	1·89	6·80	752	−65	687	817	2·50	7·56	399	−11	388	410	1·92	7·91
N_1K	485	5	490	480	2·11	7·48	661	−33	628	694	2·67	7·89	436	−51	385	487	2·49	8·16
N_1KC	565	67	632	498	2·19	7·48	873	14	887	859	2·72	7·37	440	−51	389	491	2·38	8·44
N_2	548	58	606	490	2·20	7·49	866	−41	825	907	2·49	7·64	386	−4	382	390	1·66	6·86
N_2C	581	62	643	519	1·87	6·83	807	−27	780	834	2·36	6·90	506	5	511	501	1·82	7·17
N_2K	727	51	778	676	2·53	7·95	970	0	970	970	2·84	8·77	562	−34	528	596	2·48	8·24
N_2KC	595	5	600	590	2·54	8·39	1015	−118	897	1133	3·43	8·99	597	−44	553	641	2·72	8·60

* for explanation of symbols see Part I (preceding paper)

The AA, TA and VA figures generally were increased by both nitrogen and potassium treatments. The highest values were obtained in samples from the plots which had received heavy dressings of nitrogenous fertiliser as well as potassium. The AA, TA and VA values for the second cut (August, 1956) were very high compared with those for the other two cuts.

Negative EA values were obtained in samples from most treatments at the second and third cut. The lowest values in these cuts occurred in the grass which had received both nitrogen and potassium. In the first cut only one negative EA value occurred and while the effect of nitrogen was irregular, potassium reduced the EA values consistently.

Application of potassium sulphate consistently increased the K/(Ca + Mg) ratio at all cuts. Some very high ratios were obtained in the second cut, particularly with potassium combined with high nitrogen treatments. The highest value (3·34) was obtained from the N_2KC plots. The (Ca + K)/Mg ratios were not consistently affected by different treatments.

The results of the farm herbage samples and details of the species are given in Table III. Samples 9–13 were taken from hill land which had not received any fertilisers.

Table III

Mineral composition and cation–anion relationships in some miscellaneous herbage samples

No.	Herbage	Ca	Mg	Na	K	Cl	P	S	AA	EA	TA	VA	K/(Ca+Mg)	(K+Ca)/Mg
		mequiv./kg. of dry matter							Cation–anion relationships					
	Samples from ' tetany ' areas													
1	Ley (mixed spp.)	240	133	35	202	110	242	156	−29	131	102	−160	0·54	3·32
2	Ley (,, ,,)	280	183	187	755	107	368	194	641	95	736	546	1·63	5·66
3	Ley (,, ,,)	300	142	35	535	93	310	156	321	132	453	189	1·21	5·88
4	Hill P.P.	285	167	30	310	158	397	219	−37	55	18	−92	0·69	3·56
5		275	183	91	781	113	484	231	528	−26	502	554	1·71	5·77
6	Ley (mixed spp.)	335	142	61	904	127	426	156	682	51	733	631	1·90	8·73
7	Ley (,, ,,)	335	158	39	614	110	465	206	337	28	365	309	1·25	6·01
8	Ley (,, ,,)	360	175	326	783	82	532	263	764	3	767	761	1·46	6·53
	'Normal ' samples													
9	*Molinia caerulea*	120	158	13	456	104	186	206	159	92	251	67	1·64	3·65
10	*Nardus stricta*	90	83	17	253	79	174	88	103	−1	102	104	1·46	4·13
11	*Festuca ovina*	105	125	30	402	79	252	125	228	−22	206	250	1·75	4·06
12	Hill P.P., mainly *Molinia, Nardus,* *Festuca* spp.	55	67	22	192	37	271	94	83	−149	−66	232	1·57	3·69
13		80	100	13	430	96	203	106	241	−23	218	264	2·39	5·10
14	Ley (mixed spp.)	230	150	30	397	93	319	156	178	61	239	167	1·04	4·18
15	Ley (,, ,,)	275	183	35	532	99	436	294	174	22	196	152	1·16	4·41
16*	P.P. (mixed sp., well fertilised)	280	142	52	919	79	397	225	667	25	692	642	2·18	8·44
17	P.P. (,, ,, , mainly *Festuca rubra*)	305	183	244	463	20	319	188	499	169	668	330	0·95	4·20

* cases of tetany reported the following season

Discussion

It has previously been mentioned that the ' normal ' farms had no previous record of hypomagnesaemic tetany. The field from which sample 16 had been obtained had received heavy dressings of fertilisers and it was reported in the year following sampling that three deaths had occurred in a herd of cows while grazing this particular field. In spite of the fact that no tetany had been recorded at the time of sampling it was obvious from the subsequent events that this sample was not ' normal '. The abnormality of this sample is shown in the high AA, TA and VA values as compared with Brouwer's normal range. The K/(Ca + Mg) ratio is relatively high and approaches Verdeyen's critical level of 2·20.

With the exception of the above sample most of the AA and VA values fall within Brouwer's normal limits although the TA values with one exception are abnormally low. Among the ' normal ' samples, four show negative values for EA. These all occur in hill species (*Nardus* and *Festuca* spp.).

With the exception of sample 16 referred to above, all the (K + Ca)/Mg values are below 5·2 whereas most of the samples from ' tetany ' areas show values above this level. On the other hand, the K/(Ca + Mg) ratios are generally lower in the ' tetany ' than in the normal samples. No values approaching 2·2 occur in the ' tetany samples ' and it is clear that this ratio is no indicator in the small number of samples examined.

Many of the grasses obtained from tetany-inducing pastures were characterised by high AA values in agreement with Brouwer's observations. The TA and VA values showed similar trends to the AA figures although few were abnormally high according to Brouwer's limits. Only one negative EA value occurred in grass from the ' tetany ' areas.

It is obvious that since only a few farm samples have been examined it is impossible to draw any definite conclusions from the results and it is clear that a much wider survey of tetany and normal areas in this country is necessary.

Table II shows the anion–cation relationships for the experimental samples of perennial ryegrass. The most outstanding features of these results are the exceptionally high AA, TA and VA values which occurred in most of the samples. It is difficult to explain why many of these values should be high in the control and the non-fertilised ryegrass–clover plots when compared with Brouwer's[1a] and Brandsma's[5] normal values. Possibly the fact that the present analyses were confined to a single species of grass, whilst the Dutch workers' normal values were obtained from mixed species, may be an important factor. There are few data available for the mineral composition of different species of grasses although it is clear from the work of Thomas & Thompson[7] that considerable variations between species can occur.

The majority of the EA values fall below Brouwer's lower limits for normal herbage and in the second cut all these values were negative which, according to Brouwer,[1a] is characteristic of tetany-inducing pastures. The high nitrogen–potassium treatments consistently produced the highest AA, TA and VA values at all cuts. These values were particularly high at the second cut. It is interesting to note that these high values are not necessarily associated with low herbage-magnesium content since the second cut gave samples with considerably higher magnesium content than the other two cuts (Table I). Although the high nitrogen–potassium treatments generally gave low EA values, the results were not as consistent as the other values. Table III shows that the EA values from 'tetany' and 'normal' areas are erratic. The calculation of EA does not take potassium directly into account and this may be responsible for the apparent lack of significance of this value.

The majority of the K/(Ca + Mg) ratios are above Verdeyen's limit[2] of 2·2. According to this worker and Kemp & t'Hart[3] these samples of herbage would tend to induce tetany in stock. All the values in the second cut, except the control, were above 2·2. The highest levels at all cuts were obtained on the herbage from the high nitrogen-potassium plots.

The (K + Ca)/Mg ratios in the farm samples from tetany areas (Table III) were generally higher than in the normal samples and in fact this ratio appears to be a better guide than the K/(Ca + Mg) ratio. In the experimental samples the (K + Ca)/Mg ratios were all high and were generally increased by potassium fertiliser.

Conclusions

From a consideration of a number of anion–cation relationships calculated from the results of the previous paper on perennial ryegrass it has been shown that

(1) AA, TA and VA values were increased by both nitrogen and potassium fertiliser treatments. A large number of negative EA values were obtained.

(2) The K/(Ca + Mg) ratio was consistently increased by applications of potassium and nitrogen.

(3) In a number of herbage samples taken from 'tetany' and 'normal' farms, none of the K/(Ca + Mg) ratios from the 'tetany' samples approached 2·2. The (K + Ca)/Mg ratios, however, generally gave a better indication of tetany-inducing herbage.

(4) Most of the samples obtained from tetany areas were characterised by high AA values. The TA, VA and especially EA values were less conclusive.

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EQUIPMENT FOR THE SEPARATE COLLECTION OF FAECES AND URINE FROM SHEEP

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A NUMBER of metabolism crates for the separate collection of faeces and urine from sheep have been described by various workers. These fall into two main categories, collection of excreta by bag and funnel of the pattern described by Raymond, Harris and Harker (1953), and separation without harness by the use of wire screens which deflect the faeces into a container and permit the urine to flow through a metal funnel into a collection bottle. Bratzler (1951) has described in detail a metabolism crate of this latter type which has the advantage that it can be used for sheep of either sex.

In a number of metabolism studies carried out on wether sheep with fresh pasture grass and silage it has been found that because of the high moisture content of faeces resulting from succulent diets of this type, the most satisfactory system of faeces-urine separation is by bag and harness. A metabolism crate and harness which has been in use over a period of years in fresh grass and silage feeding experiments is described below. This equipment has also been used satisfactorily for calcium and magnesium balance studies at the Moredun Institute (Butler and Field, private communication).

Crate. The crate (Plates 1 and 2) is built of wood and accommodates two sheep, the two being separated by a double partition of hardboard except for a small wire mesh window (18 in. × 12 in.) at head level. The internal dimensions of each half of the crate, excluding feeding-box, are 62 in. × 20 in. The aluminium-lined feeding-boxes are completely enclosed and are easily removed by releasing two small bolts attached to the front side of the crate. The sheep are removed from the crate by means of a ramp; entrance to the crate is made at the rear end using the same ramp. The sides of the crate are also removable for harness adjustment or easy access to faeces bag. The floor of the crate is constructed of wood and covered with a layer of $\frac{1}{8}$ in. rubber sheeting. During experiments the animals are held by means of a loose chain attached from the front of the crate to a leather collar. Sufficient freedom of movement is allowed to enable the animal to feed and rest in comfort. Water troughs are fixed on the outside of the crate, the hood over the trough being fitted with a wire mesh guard.

Faecal collection. The harness for the collection of faeces is shown in Plate 4. The faeces is collected in a polythene bag which is supported in a strong canvas outer bag which contains a stiff metal ring sewn into the opening. The method of attachment of this outer bag is a modification of that devised by Dijkstra (private communication.) A cylinder of canvas ($6\frac{1}{2}$ in. diameter $\times$ 3 in. wide) fixed to the rear end of the body harness contains five loops of webbing through which pass a leather strap. The cylinder of canvas fits into the faeces bag which is then clamped firmly in position by tightening and buckling the leather strap. By weighing the polythene bag empty and the following day with contents, an accurate measure of total fresh faeces can be obtained. The use of a polythene bag eliminates any errors and inconveniences caused by transferring wet fresh faeces from the collection bag into a weighing receptacle.

Urine collection. The urine funnel (Plate 3) is made of rubber and is held in position by a separate canvas support. Four straps attached to the support pass through slits in the rim of the funnel then encircle the animal's body. The tube from the funnel passes through a hole in the side of the crate and into a five-litre polythene receiver.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. E. J. Butler and Mr. A. C. Field for their suggestions.

The harness was made by J. H. Rogers and Co., 122A George Street, Edinburgh, and the rubber urine funnels by W. B. Hilliard and Sons Ltd., 123 Douglas Street, Glasgow, C. 2.

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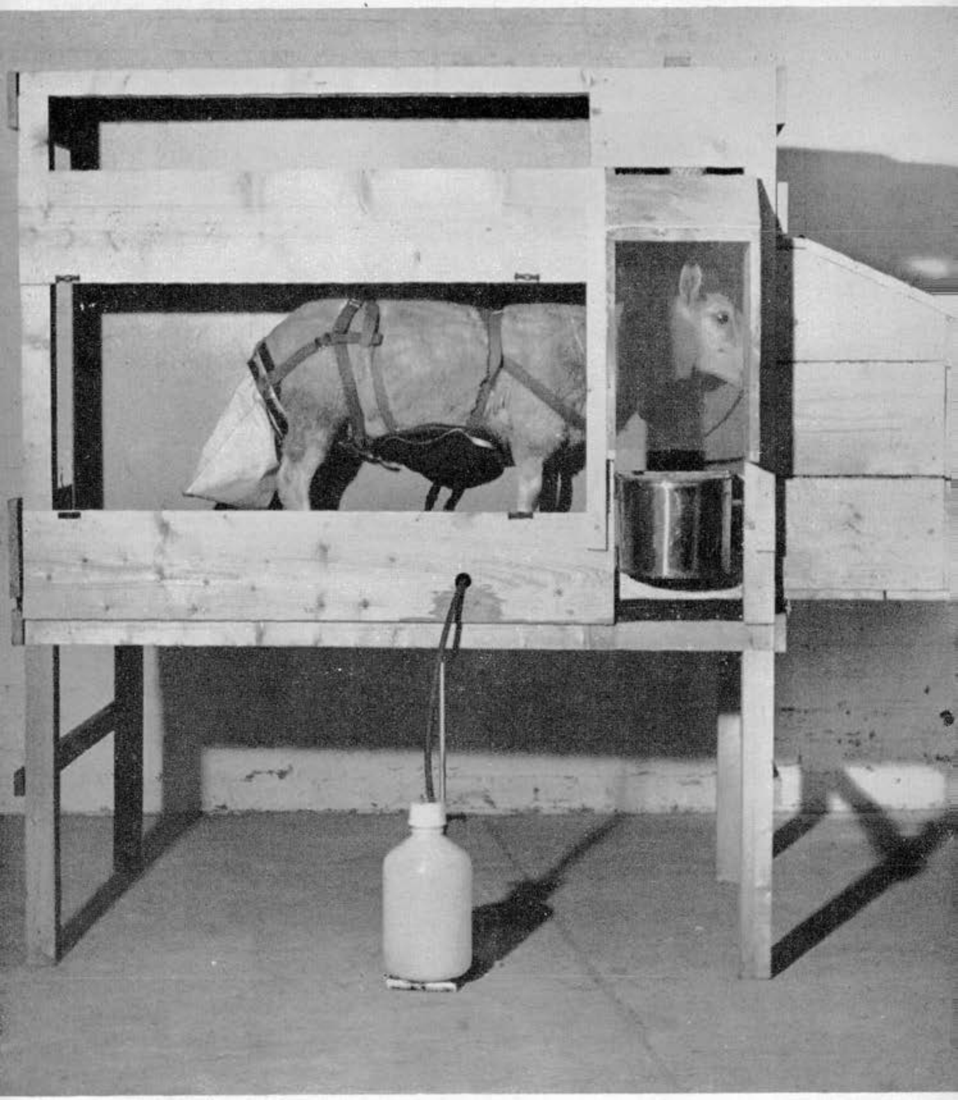
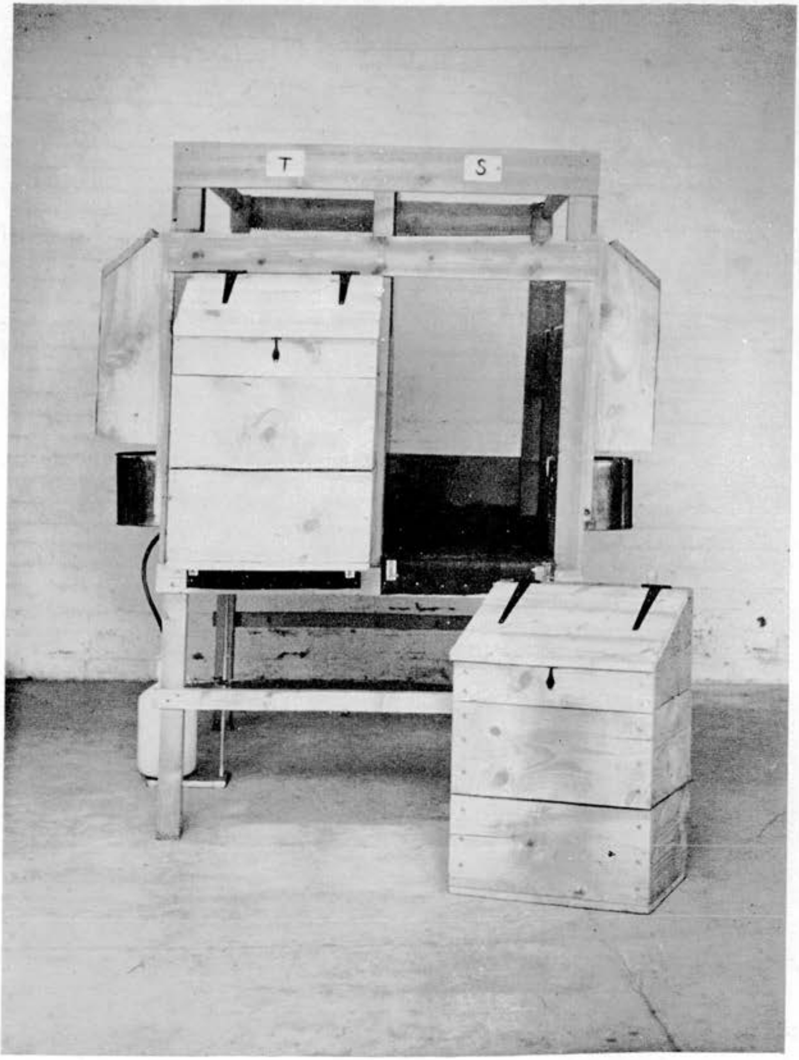
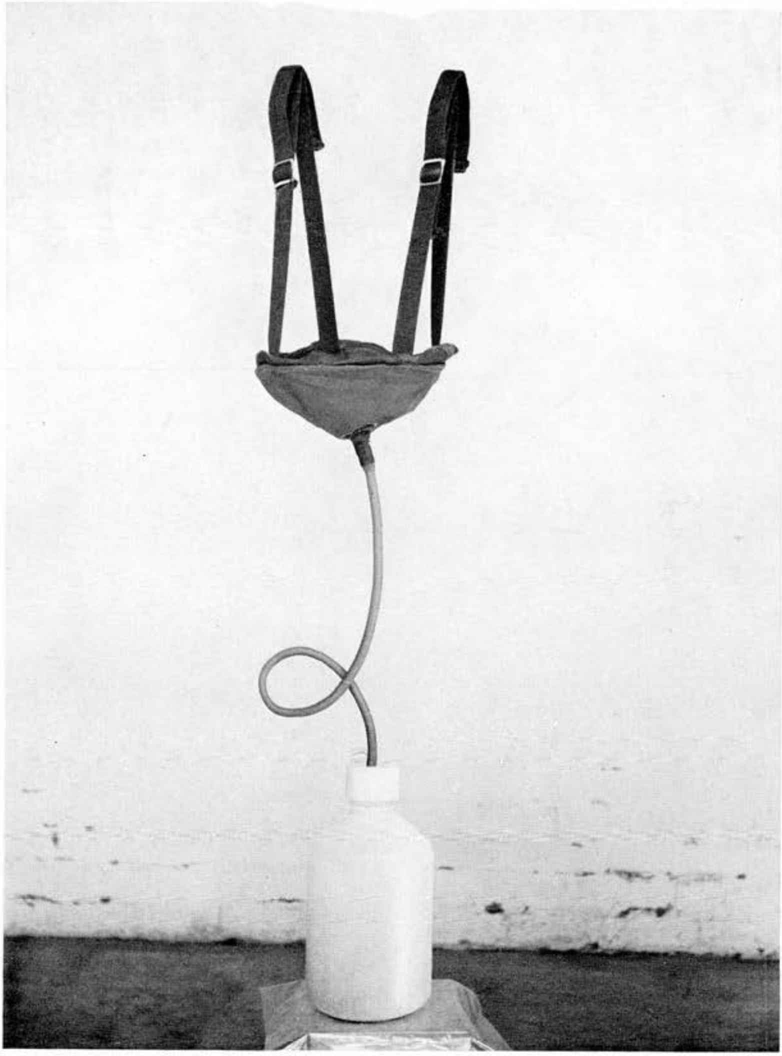


Side view of crate with outer side removed to show interior.



Front view of crate with right feeding box removed.



Urine funnel with canvas support.

PLATE 4

Harness with faeces bag detached.

25.

The effect of early weaning on the blood sugar and rumen acid levels of the growing calf

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The dry feeding of young calves, 'early weaning', has become the established practice on many farms. The system described by Preston (1957) consists in abruptly replacing the milk diet of the calf, at 3–4 weeks of age, with dry palatable concentrates which are first given to the animal when it is 7 days old. Because the level of milk given is restricted to about 6 pints daily the calf is encouraged to consume appreciable amounts of concentrates before it is weaned.

The favourable results obtained by Preston (1956) with this system have been confirmed by others. Though it is believed that the beneficial effects of the system result from the rapid establishment of adult rumen functions in the young animal, there is a lack of experimental appraisal of the development of these functions and of their effect on the animal's metabolism.

The purpose of this investigation was to study the effect of early weaning on the amounts of volatile fatty acids and lactic acid in the ruminal fluid and on the blood sugar levels of the growing calf.

EXPERIMENTAL

Animals and treatment. Twelve Ayrshire bull calves, 3–7 days old, were used in two experiments, each lasting for 84 days. In each experiment six animals were divided equally between two feeding treatments: control and early weaning. The control calves received milk throughout the experimental period, and milk was withdrawn from the diet of the early-weaned calves at 28 days. Both groups were offered concentrates from 10 days of age.

Feeding and management. The calves were housed indoors at The Edinburgh School of Agriculture dairy farm and kept in individual pens littered with sawdust. The animals were weighed and measured on the 2nd and 3rd days after purchase and allotted to the treatments. For each calf the means of the weights and measurements on both days were taken to represent the live-weight and body measurements at 3 days of age. Regular weighings were taken at 14-day intervals.

From the 2nd day all the animals were given liquid whole milk in amounts which were gradually increased for each calf over 3 days to the full daily allowance based on the standards of Roy, Shillam, Hawkins & Lang (1958).

The animals in the control group were offered milk to provide for maintenance plus 1 lb live-weight gain/day and the early-weaned calves were given milk sufficient for maintenance plus 0·5 lb live-weight gain/day. The difference was intended to induce the experimental animals to take more readily to the concentrates offered to all the calves from 10 days of age.

The composition of the concentrate mixture was based on the early-weaning mixture described by Preston (1958) but was modified by the inclusion of dried grass. The percentage composition of the mixture was: flaked maize 36, bruised oats 22·5, molassine meal 13·5, soya-bean meal 4·5, fish meal 9·0, stabilized tallow 4·5, salt 0·32, vitamins A and D supplement 0·4, Aurofac 2A (Cyanamid Ltd) 0·2, grass meal 9·08.

The concentrate mixture was given to appetite, in two equal portions at 8 am and 4 pm. Refusals were weighed and discarded. From 10 days of age water was also offered *ad lib.*

During the first 4 weeks, the calves in Expt 1 were given dried whole milk reconstituted with eight times its weight of warm water. The use of this milk was discontinued when facilities for warming fresh milk were available.

Sampling. Samples of blood and rumen liquor were taken at 14-day intervals from each calf before it was fed and 1, 2, 3 and 4 h after feeding. Blood samples were withdrawn from the jugular vein by the technique of Preston & Ndumbe (1961) with heparin as anticoagulant. Samples of rumen liquor were obtained with a stomach tube and vacuum pump. In some instances the 3rd h postfeeding sample was not taken.

Before analysis, all samples were stored in a deep-freeze refrigerator at $-13°$.

Analytical methods. The concentration of reducing sugars in the blood was determined by the method of Somogyi (1952) with the colorimetric reagents of Nelson (1944).

Total volatile fatty acids in rumen liquor were determined by distillation after filtration, acidification and protein precipitation by the method described by Elsden, Hitchcock, Marshall & Phillipson (1946).

In Expt 1, selected samples of rumen liquor were analysed for individual volatile fatty acids by means of the Celite column chromatographic method described by Wiseman & Irvin (1957). The samples selected for detailed acid analysis were the prefeeding sample and the sample at peak volatile fatty acid production, taken at 28 and 84 days of age. Lactic acid in rumen liquor was determined by the method of Barker & Summerson (1941).

RESULTS

Health of animals. All the calves in Expt 1 scoured during the 1st month. The incidence was more severe in the control animals, and treatment with streptomycin and sulphadimidine (BP) had little effect. Scouring ceased as soon as powdered milk was replaced by fresh milk.

One calf (K 95) in the early-weaned group of Expt 1 died of pneumonia in the 6th week. Apart from this animal, the health of all the calves appeared to be normal.

Growth and feed intake. The overall mean values of live-weight gain and body measurements are shown in Table 1. The early-weaned calves ate the concentrate mixture readily, and before weaning, at 28 days, they were consuming about ½ lb of it (air-dry)/head daily. Growth was slow in both the experimental and control groups during the period of scouring from 3 to 14 days of age when mean gains of 1·3 and

2·5 lb/head occurred. No significant differences between treatments were found in live-weight gains over the 84-day period. The control animals, however, gained significantly more ($P = 0·01$) in live weight during the period 28–42 days. With respect to skeletal growth from 3 to 84 days, significant differences in favour of the control calves were observed in height at withers ($P = 0·05$) and in width of hooks ($P < 0·05$).

Table 1. *Growth and feed intake of six control and six (five)† experimental calves from 3 to 84 days of age*

Measurement	Control	Early-weaned	SE of difference
Weight at 3 days (lb)	75·0	71·6	—
Feed consumption, 3–84 days (lb/head):			
Liquid whole milk	771·0	163·5	—
Concentrate ration	62·7	177·2	—
Gain in weight (lb/head)			
3–14 days	2·5	1·3	± 2·60
14–28 days	9·1	11·7	± 3·56
28–56 days‡	32·0	25·9	± 4·78
56–84 days	47·9	36·7	± 5·10
3–84 days	91·5	75·6	± 10·64
Gain in body size, 3–84 days (cm/head):			
Height at withers	12·0	9·7	± 1·00*
Length	18·0	14·3	± 2·35
Heart girth	22·1	18·2	± 1·93
Middle girth	39·3	40·3	± 3·78
Width of hooks	6·1	4·2	± 0·63*

* Significant at $P < 0·05$.

† Calf K95 died 6 weeks after the beginning of the first experiment.

‡ Live-weight gains (lb) during the period 28–42 days for individual animals were: control 17·7, 20·5, 15·5, 24·0, 6·2, 19·0; early-weaned 3·0, 2·7, 5·5, 10·7, 10·2, 7·0.

Changes in blood sugar concentration. The mean blood sugar levels for the two experiments before and after feeding are shown in Fig. 1. During the first 4 weeks when all the animals were getting milk and concentrates, there was little difference between treatments. In both groups the blood sugar levels increased markedly 1 h after feeding and then declined rapidly during the next 2 h. At 6 weeks, that is 2 weeks after early weaning of one group, the prefeeding blood sugar values of the early-weaned calves decreased markedly below the levels at 4 weeks (a fall from 81·4 to 69·3 mg/100 ml) and remained below the corresponding levels of the control animals over the remainder of the experimental period. Blood sugar values of the control animals continued to show marked fluctuations throughout the experiment, but similar changes were not observed in the early-weaned calves after feeding from the 6th week.

Volatile fatty acids and pH of rumen contents. The total volatile fatty acid (VFA) content and pH of the rumen liquor samples are shown in Table 2. The peak values for VFA for the two groups are shown in Fig. 2.

The total VFA content of the rumen liquor increased with age more rapidly for the early-weaned animals than for the control animals. In the early-weaned group, peak concentrations were relatively stable at 6–8 weeks but the values for the control group continued to increase up to 12 weeks. Except at the beginning of the experiment, the levels of VFA were higher in the early-weaned than in the control calves, the most striking difference occurring at 6 weeks. In both groups peak concentrations usually occurred 2–3 h after feeding.

The mean pH values of the rumen liquor samples from both groups did not differ significantly.

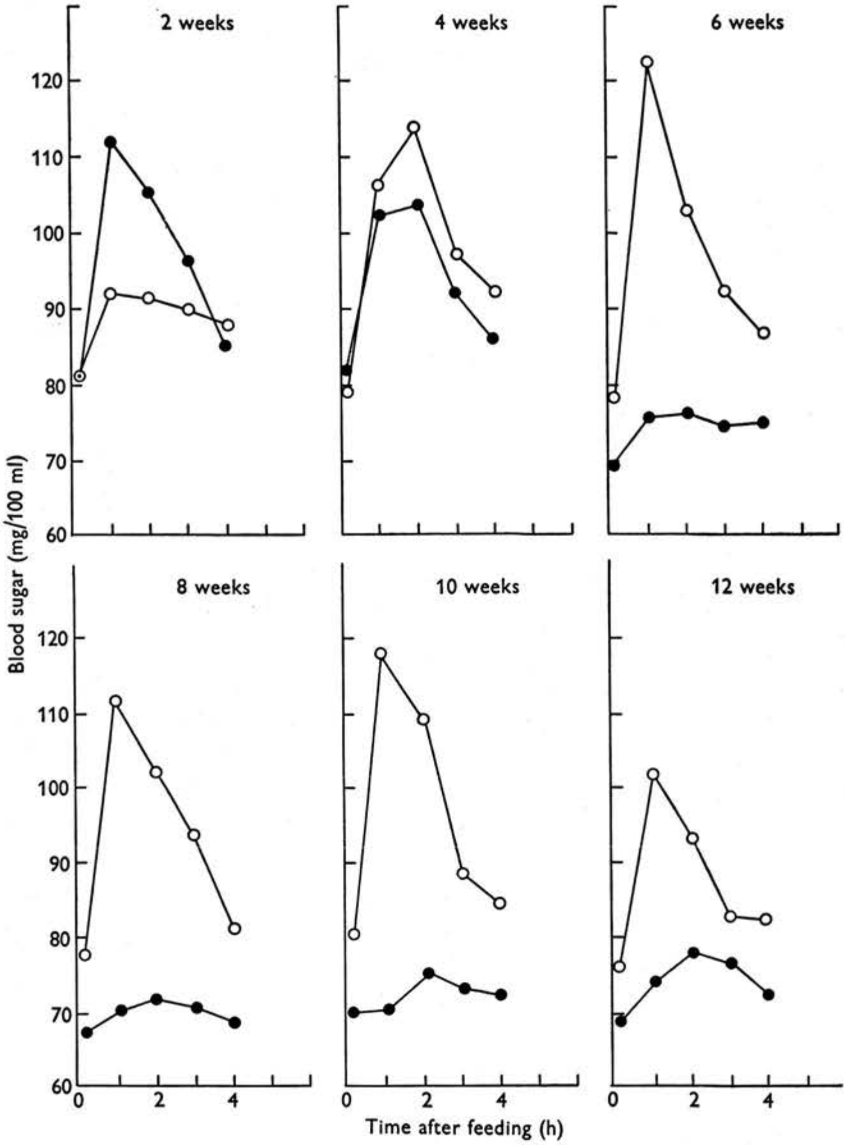

Fig. 1. Mean concentration of blood reducing sugar of control (○—○) and early-weaned (●—●) calves at different ages.

Table 3 shows the amounts of the individual VFA present in some of the rumen liquor samples. The detailed analysis of the acids was carried out in Expt 1 only, and it was done only on prefeeding samples and on those showing peak VFA values.

The total VFA values for twenty-one samples determined by the distillation method agreed reasonably well with the sum of the individual acids determined by the Celite column chromatographic method. Recovery of VFA from the column was 98·4% (SE ± 0·94).

Although the results suggest that there was little or no difference between treatments in the relative proportions of acetic, propionic and butyric acids at 4 and 12

Table 2. *Difference in mean values for concentration of ruminal total volatile fatty acids (VFA) and pH between the early-weaned and the control calves*

Measurement	Week	Control	Early-weaned	Difference	SE of difference	Significance
VFA concentration (m-moles/l.)	2	78	71	− 7	± 10·3	NS
	4	90	119	+29	± 11·6	$P < 0·05$
	6	100	144	+44	± 8·0	$P < 0·001$
	8	120	137	+17	± 18·3	NS
	12	126	146	+20	± 8·6	$P < 0·05$
pH	2	5·53	5·35	− 0·18	± 0·28	NS
	4	5·58	5·32	− 0·26	± 0·24	NS
	6	5·42	5·17	− 0·25	± 0·12	NS
	8	5·43	5·24	− 0·19	± 0·28	NS
	12	5·48	5·36	− 0·12	± 0·13	NS

NS, not significant.

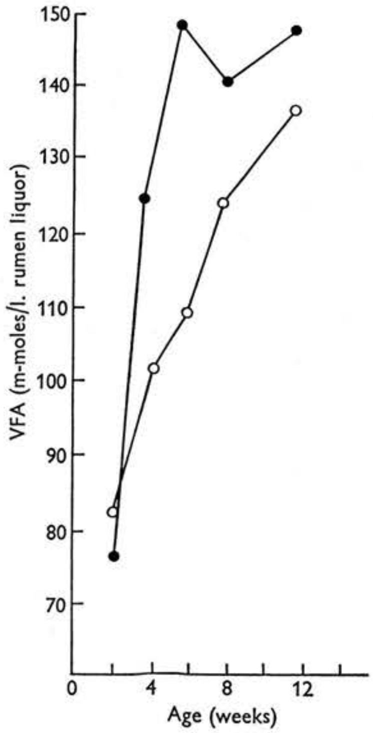

Fig. 2. Total volatile fatty acids (VFA) (peak values) in the rumen of control (○—○) and early-weaned (●—●) calves at different ages.

weeks, there was some indication that the proportion of butyric acid in the early-weaned calves was lower at 12 weeks than at 4 weeks.

Lactic acid production in the rumen. The ranges in concentration of lactic acid in the rumen liquor samples are given in Table 4. Apart from a few instances in which high levels of lactic acid were found in the liquor sample taken before feeding, the pre-feeding concentration generally ranged from 0 to 5 mg/100 ml. This value increased markedly 1 h after feeding. One calf in the early-weaned group which showed a high prefeeding level (68·5 mg/100 ml) at 8 weeks also exhibited distress, lassitude and anorexia. The signs were transitory and the animal had apparently recovered by the next day.

Table 3. *Individual volatile fatty acids (VFA) in the rumen liquor of calves in Expt 1 at 4 and 12 weeks*

			Total VFA (m-moles/l.)		Butyric acid (% VFA)		Propionic acid (% VFA)		Acetic acid (% VFA)	
Week	Treatment	Calf no.	Pre-feed	Post-feed-	Pre-feed	Post-feed	Pre-feed	Post-feed	Pre-feed	Post-feed
4	Control	K 94*	58	62·8	19	19	23	30	58	51
		K 97*	31	99·9	21	18	30	28	49	54
		K 100	92	No sample	21	—	33	—	46	—
		Mean	60	81·4	20	18	29	29	51	52
4	Early weaning	K 95*	98	139	19	16	28	38	52	45
		K 98*	115	132	20	19	34	33	47	48
		K 99†	105	146	16	17	28	32	56	50
		Mean	106	139	18	17	30	34	52	48
12	Control	K 94‡	192	290	24	22	32	34	44	44
		K 97†	161	317	16	22	38	34	46	44
		K 100†	121	190	25	21	32	31	42	48
		Mean	158	266	22	22	34	33	44	45
12	Early weaning	K 98*	163	281	19	17	30	34	51	49
		K 99*	198	303	12	13	24	32	64	55
		Mean	180	292	15	15	27	33	55	52

* Sample taken 3 h after feeding. † Sample taken 4 h after feeding. ‡ Sample taken 2 h after feeding.

The lactic acid values showed considerable variation between individual animals, making a statistical comparison valueless.

The relationship between VFA, lactic acid and pH at 2, 6 and 12 weeks for the two experiments is shown in Fig. 3. The levels of total VFA appeared to be inversely related to the pH.

DISCUSSION

It is known from previous work with calves (Preston & Ndumbe, 1961) that blood sugar concentration increases after a feed of milk and then falls rapidly to normal levels. It has been shown also that the rate of flow of liquid milk from the abomasum to the duodenum is fairly rapid (Barhydt & Dye, 1957) and that virtually all the milk lactose is quickly digested and absorbed from the small intestine and rapidly utilized by the animal (Davis & Brown, 1962). In view of these findings the changes in blood sugar concentration that occurred in our studies were to be expected.

Table 4. *Concentration (mg/100 ml) of lactic acid in the rumen liquor of calves*

Week	Time after feeding (h)	Control Range	Control Mean	Early-weaned Range	Early-weaned Mean
2	Prefeeding	0·2–14·5	8·5	0·5– 3·2	2·0
	1–2	1·8–44·2	18·1	11·7–38·1	25·4
	4	0·0–13·2	5·6	2·3–48·0	14·5
4	Prefeeding	0·0– 9·0	3·0	0·2– 9·0	2·7
	1–2	1·1–27·1	7·5	2·0– 7·7	5·4
	4	0·3–13·5	5·1	1·6–14·4	4·9
6	Prefeeding	0·4–20·2	6·3	0·7–21·5	8·6
	1–2	1·0–13·6	7·4	6·4–63·2	19·9
	4	0·7–19·8	9·3	4·6–29·9	13·1
8	Prefeeding	3·4–14·5	8·5	4·9–68·5	20·4
	1–2	3·1–31·9	19·4	1·5–64·3	33·2
	4	2·5– 7·2	4·2	2·4– 8·7	5·3
12	Prefeeding	0·0– 6·0	2·5	1·0– 4·0	3·1
	1–2	4·6–40·6	17·2	14·9–56·3	36·0
	4	0·0– 5·8	4·1	1·0– 5·6	4·2

A marked decline in the blood sugar levels occurred in the early-weaned animals at 6 weeks, that is 2 weeks after weaning. Hibbs, Conrad & Pounden (1952) found a similar decline in blood sugar in calves at 7 weeks when the calves were given roughage diets.

It is well known that blood sugar levels in young ruminants decline steadily with age. Reid (1953) demonstrated that a decline in the postabsorption blood glucose level in lambs began in the 1st week of life, that a large proportion of the decline was due to the disappearance of glucose from the corpuscles, and that plasma glucose level began to decline rapidly at about 4–5 weeks of age to reach stable adult levels between the 7th and 9th weeks. Hibbs *et al.* (1952) observed that much of the decline in blood glucose level of calves during the postnatal 7-week period of milk feeding resulted from a decrease in corpuscle glucose. Plasma glucose level declined markedly only after withdrawal of milk from the diet. These workers also found that the level of plasma glucose in animals given antibiotics was higher than in control animals. In our studies the sharp fall in total blood sugar level which occurred at 6 weeks may therefore be attributed largely to a decline in plasma glucose level because of abrupt withdrawal of milk from the diet, depriving the animal of a readily available supply of sugar. The relatively high blood sugar levels in all groups and the slow decline in blood sugar level with age, especially in the control animals, may be attributed to the inclusion of an antibiotic in the concentrate supplement, which according to Hibbs *et al.* (1952) deters fermentation in the rumen by depressing the activity of certain bacteria. At 6 weeks, the blood sugar values in the early-weaned calves did not show any marked fluctuation after feeding.

It is clear from the total VFA values shown in Table 2 and Fig. 2 that fermentation in the rumen was developed more rapidly in the early-weaned animals than in the control animals because of the ingestion of relatively large amounts of concentrates at an early age.

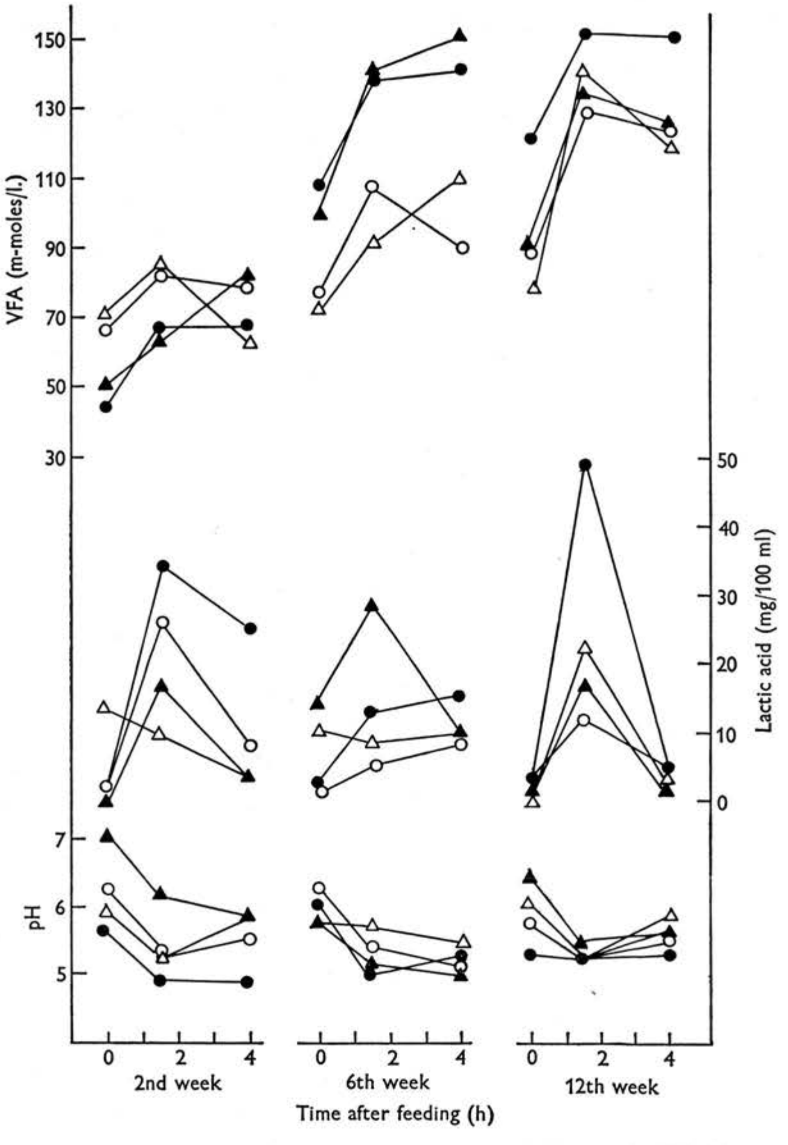

Fig. 3. Relationship between pH, lactic acid and total volatile fatty acids (VFA) in the rumen liquor of control and early-weaned calves at different ages. Expt 1: △—△, control; ▲—▲, early-weaned calves. Expt 2: ○—○, control; ●—●, early-weaned calves.

With regard to the mixture of the ruminal acids, the relatively high proportion of propionic and butyric acids and the correspondingly low levels of acetic acid observed in both the control and the early-weaned calves agree with previous results obtained with sheep (Phillipson, 1952) and dairy cows (Balch & Rowland, 1957) given diets rich in soluble carbohydrates.

The postfeeding distribution of the rumen fatty acids at peak values indicates in general that an increase in the proportion of propionic acid occurred with a corresponding decrease in acetic acid. Stewart, Stewart & Schultz (1958) and Reid, Hogan & Briggs (1957) reported that propionic acid is produced faster than butyric or acetic acid and that peak propionic acid values coincide with the peak VFA concentrations. The high concentration of ruminal lactic acid in both groups after feeding substantiates the results of Phillipson (1952) and Balch & Rowland (1957) and was due to the rapid fermentation of soluble carbohydrates. It is also probable that the presence of antibiotics stimulated lactic acid production (Dinda, 1960).

With regard to the relative effects of lactic acid and VFA on the rumen pH, Briggs, Hogan & Reid (1957) studied this relationship in sheep on a wide range of diets and concluded that rumen pH is largely a function of rumen VFA level. Lactic acid exerts a stronger effect than VFA and is associated with considerably lower pH levels than would be recorded in its absence.

Our results also suggest that there is an inverse relationship between total VFA and pH over a fairly wide range of values and that low pH values are associated with relatively high concentrations of lactic acid in the rumen.

SUMMARY

1. Changes in blood sugar level and in the concentrations of volatile fatty acid (VFA) and lactic acid in rumen liquor were studied in two groups of six Ayrshire bull calves from 3 to 84 days of age. One group was weaned from milk at 28 days; the other continued to receive milk for 84 days.

2. There was no significant difference between the groups in body-weight over the 84-day period although significant differences in favour of the control calves were observed in height at withers and in width of hooks.

3. The blood sugar concentrations of the early-weaned calves fell markedly after weaning whereas the values for the control animals steadily declined with age.

4. The levels of total VFA in rumen liquor increased with the age of the calves and were significantly higher at 6 weeks in the early-weaned group than in the late-weaned group.

5. In both groups, the VFA contained a relatively higher proportion of propionic acid and a lower proportion of acetic acid than is commonly found with adult ruminants on diets containing adequate amounts of roughage.

6. The concentration of lactic acid in the rumen liquor of the animals in both groups increased immediately after feeding but great variation occurred in the amount of the increase.

7. The results indicate that adult blood and rumen characteristics can be induced in the calf at 6 weeks of age by the early withdrawal of milk from the animal's diet.

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